Characterisation of a novel culture condition for the establishment and maintenance of mouse embryonic stem cells and implications for the mechanisms of self-renewal.

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Abstract

Pluripotency is defined as the ability of a cell to give rise to all the cell types of the adult organism. *In vivo* this property is possessed transiently by the cells of the epiblast in the developing embryo but it can be maintained indefinitely by deriving embryonic stem (ES) cells. How the pluripotent state is established in the cells of the early embryo and how it is 'captured' and maintained in the form of ES cells is a fascinating question for biology with practical implications. It is hoped that ES cells will be of use in biomedical research and cell replacement therapy. Our understanding of their biology and our ability to manipulate the cells *in vitro* will be of great importance if these hopes are to be realised.

The starting point for the work presented in this thesis was the development of a novel culture condition for the derivation and maintenance of mouse ES cells (Q-L. Ying and J. Nichols). The media is formed by the addition of three small molecule inhibitors to a previously described serum-free media, N2B27, and is termed 3i (three inhibitors).The inhibitors are SU5402, PD184352 and CHIRON99021, and they inhibit the FGF receptor, mitogen activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK), and glycogen synthase kinase 3 (GSK3) respectively.

I attempt to further our understanding of pluripotency and self-renewal in ES cells by genetic and biochemical examination of ES cells cultured in 3i. Analysis of intracellular signalling pathways together with descriptions of genetic mutants for the targets of the inhibitors validates the mode of action and the specificity of the three inhibitors. Self-renewal of mouse ES cells is considered dependent on activation of STAT3 through provision of the cytokine leukaemia inhibitory factor (LIF). I demonstrate unequivocally that this pathway is not required for self-renewal in 3i by characterising *Stat3*-null ES cells. Further experiments reveal that preventing activation of ERK downstream of the growth factor FGF4, produced by the ES cells themselves, is key to preventing differentiation. Pleiotropic effects of GSK3 inhibition are observed and candidate GSK3 targets with known or predicted effects on self-renewal are investigated as potential downstream effectors. I propose that activation of canonical Wnt signalling, together with a global derepression of biosynthetic capacity, mediate the pro-self-renewal effects of GSK3 inhibition.

The description of culture conditions that function independently of signalling pathways previously thought essential for self-renewal provides fresh insight into the nature of ES cell self-renewal and the relationship of ES cells to the pluripotent cells of the developing embryo. There are practical implications for ES cell biology as there is reason to hope that the new conditions will translate more readily to other mammalian species to facilitate the derivation of ES cells and will provide an optimal platform for differentiation of ES cells into somatic cell types of interest.

"There is a theory which states that if ever anyone discovers exactly what the universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

"There is another theory which states that this has already happened."

Douglas Adams
The Restaurant at the End of the Universe
The Hitchhiker's Guide to the Galaxy

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My parents' support and their blind belief in my capabilities made it possible for me to complete my first degree and to continue to study for my PhD. Thank you.

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Abbreviations

2i	two inhibitors	m	mouse
3i	three inhibitors	mRNA	messenger RNA
4OHT	4-hydroxytamoxifen	MAPK	mitogen activated protein kinase
BMP	bone morphogenic protein	MEFs	Mouse embryonic fibroblasts
ChIP	chromatin IP	maGSC	multipotent adult germline stem cell
CHIR	CHIRON99021	mGS	multipotent germline stem
cDNA	complimentary DNA	PBS	phosphate buffer saline
dpc	days post coitum	PAGE	polyacrylamide gel electrophoresis
DNA	deoxyribonucleic acid	PCR	polymerase chain reaction
DMSO	dimethyl sulphoxide	PD03	PD0325901
EC	embryonal carcinoma	PD17	PD173074
EG	embryonic germ	PD18	PD184352
EGF	epidermal growth factor	PGC	primordial germ cell
EpiSC	Epiblast stem cell	PI3K	phosphatidylinositol 3-kinase
ES	embryonic stem	PKB	protein kinase B
ERK	extracellular receptor kinase	PrE	primitive endoderm
FGF	fibroblast growth factor	PS	PD18 plus SU
FGFR	FGF receptor	qRT-PCR	quantitative real-time PCR
FITC	fluorescin isothiocyanate	RNA	ribonucleic acid
FCS	foetal calf serum	siRNA	short interfering RNA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	shRNA	small hairpin RNA
GCNF	germ cell nuclear factor	SB21	SB216762
GDF	growth differentiation factor	SB41	SB415286
GFP	green fluorecent protein	SDS	sodium dodecyl sulphate
h	human	sIL-6R	soluble IL6 receptor
ICM	inner cell mass	SH2	src homology 2
IL-6	interleukin 6	SOCS	suppressor of cytokine signalling
iPS	induced pluripotent stem	SSC	spermatogonial stem cell
IRES	internal ribosomal entry site	STAT	signal transducer and activator of transcription
JAK	janus activated kinase	SU	SU5402
Klf	Kruppel-like family	TE	trophectoderm
lacZ	beta-galactosidase	Tet	tetracycline
LIF	leukaemia inhibitory factor	TF	transcription factor

I declare that the work presented in this thesis is my own, unless otherwise stated. The work described in this thesis has not been submitted for any other degree or professional qualification.

JASON WRAY

Chapter 1: General Introduction

1.1 What are Stem Cells and why should we study them?

Stem cells are defined by two properties: long-term self-renewal and the ability to differentiate (Gardner and Beddington, 1988). Self-renewal is the division of a cell to give rise to two daughter cells, at least one of which is identical to the cell from which they arose. Differentiation occurs when the stem cell becomes a more specialized cell type and loses the capacity for self-renewal. Stem cells can give rise to one or more distinct cell types. These properties make stem cells extremely interesting in the study of development, disease and cancer.

Stem cells have attracted a large amount of attention in recent years because it is believed that they will one day provide a limitless source of tissue for the treatment of degenerative disease (Gardner, 2007). Diseases such as Type 1 diabetes and Parkinson's disease result from the loss of specialized cell types and a subsequent loss of function in the affected tissue. It is hoped that science will be able to recreate the lost cells *in vitro* and so provide an unlimited source of cells for replacement therapies. While this application of stem cells is extremely exciting the realisation is a long way off. It has proved extremely difficult to impose one particular cell fate on cells removed from their natural environment and issues such as immune rejection would create difficulties for transplant patients. However, for scientists the attraction of stem cells goes beyond the headline. Stem cell biology is the study of how cells make cell fate decisions. That is, how they decide whether to continue dividing as a stem cell (self-renewing) or to differentiate into a specialized cell type. Our understanding of these cell fate decisions underlies our understanding of developmental biology and will ultimately determine our ability to manipulate stem cells to achieve our goals.

A more tangible use of stem cells is in providing model systems for drug discovery and for the study of cancer (Smith, 2001). Stem cells grown *in vitro* can provide

clinically relevant cell types on a large scale that can be used for high throughput drug screening. It is also believed that many cancers contain a population of cells that behave as stem cells (reviewed in (Reya et al., 2001)). That is, cells which retain the ability to proliferate and to give rise to the different cell types that compose a tumour. Such cells, if they are not destroyed by cancer treatments, can reinitiate a cancer. Therefore, cancer treatments designed to target the stem cell compartment of a cancer would prove more effective in long-term treatments.

Scientists broadly categorise stem cells into two subgroups: adult and embryonic. Adult stem cells persist throughout the life of an organism. They are responsible for the replacement of tissues lost during normal turnover or as a result of injury (Dor and Melton, 2004). They are tissue-restricted, generally only giving rise to cell-types of the organ in which they are found. Where they have been described they exist in a compartment of the tissue known as the niche (Zhang et al., 2003; Alvarez-Buylla and Lim, 2004; Watt et al., 2006) which is thought to provide signals that regulate stem cell replication and differentiation (Scadden, 2006). Their numbers are tightly regulated and maintained throughout the life of the organism and they are generally slow-cycling but possess the ability to respond to demand by proliferating rapidly to provide a source of cells for tissue homeostasis or repair. Stem cells residing in the adult tissues are extremely interesting for several reasons. Understanding how they are regulated *in vivo* provides insights into how the body maintains and repairs itself, the consequences if the stem cell compartment is lost or becomes deregulated and the idea that diseases might be treated by manipulating these cells in their normal environment. Furthermore, adult stem cells have been instructed during the development of the organism to acquire a restricted cell fate. Thus, they are closer to fully specialized cell types and may therefore be more readily manipulated to adopt a given cell fate if they can be isolated and expanded *in vitro*.

Embryonic stem (ES) cells are unique in their ability to differentiate into cells from each of the three germ layers, ectoderm, endoderm and mesoderm – they can make all the tissues of the adult organism (a property termed pluripotency) (Smith, 2006). This, together with their ability to multiply in culture without apparent limit, makes

them an attractive source for the *in vitro* generation of cell types that may be of use to study disease or to provide a limitless source of cells for cell-replacement therapies. ES cells differ significantly from adult stem cells in that they are derived from a cell type that exists only transiently in the expanding epiblast of the embryo. This distinction is critical because it means that ES cells are defined as an *in vitro* cell type, existing only in cell culture. The origin of ES cells in the early embryo provides a window into the processes of early development which are otherwise difficult to access but the artificial environment in which ES cells necessarily exist means inferences drawn from experiments about the developing embryo, performed on ES cells, must be interpreted with caution. It is hoped that by understanding the molecular mechanisms that regulate self-renewal and differentiation of ES cells we can gain insights into early development and eventually control the behaviour of these cells *in vitro* and hence provide cell types of clinical relevance.

1.2 Background to Embryonic Stem Cells

1.2.1 Origin of Embryonic Stem Cells

The observation that embryos grafted into mice produced teratocarcinomas (Stevens, 1968; Solter et al., 1970; Stevens, 1970) from which pluripotent stem cells could be derived gave rise to the idea that it should be possible to derive pluripotent cell lines direct from the embryo. The observation that coculture with fibroblasts supported growth of embryonal-carcinoma (EC) cells (Martin and Evans, 1975b), and that these cells retained a high differentiation capacity, was a critical step towards the first derivation direct from the embryo. In 1981 Evans and Kaufman (Evans and Kaufman, 1981), and Martin (Martin, 1981) succeeded in establishing pluripotent cell lines by plating delayed-implantation blastocysts or inner cell masses isolated from the expanded late blastocyst by immunosurgery onto a layer of mitotically inactivated STO fibroblasts. The resulting cell lines displayed characteristics of EC cells but retained a normal karyotype whereas EC cells normally display

aneuploidies. Martin termed the new cell lines embryonic stem (ES) cells to distinguish them from their tumour-derived counterparts.

ES cells were shown to derive from the epiblast, the cells that would normally go on to form the embryo proper (Fig1.1). In fact, ES cells can be derived with high efficiency if the epiblast is isolated by micro-dissection (Brook and Gardner, 1997). During preimplantation development the first lineage segregation occurs when the trophectoderm is specified. At the 8-cell stage embryos undergo compaction and cells become polarised with a clear distinction of inside to outside. Subsequent divisions establish the outer cells, the trophectoderm and the inner cells, the inner cell mass (ICM). The trophectoderm gives rise to the placenta while the ICM will give rise to the developing embryo and associated yolk sack, allantois and amnion (Rossant and Tam, 2004). These two cell populations can be distinguished by the expression of marker genes such as *Cdx2* and *Eomes* (Strumpf et al., 2005) (trophoblast) or *Oct4* (Palmieri et al., 1994; Nichols et al., 1998) and *Nanog* (Chambers et al., 2003; Mitsui et al., 2003; Chazaud et al., 2006) (ICM). The trophectoderm secretes fluid internally to form a cavity known as the blastocoel. The ICM becomes localised to one side of the cavity forming the distinctive structure known as the blastocyst. The second lineage segregation is the separation of primitive endoderm (PrE) or hypoblast and epiblast from the ICM. PrE will only contribute to extraembryonic tissues while the epiblast goes on to form the embryo itself (Gardner and Beddington, 1988). The PrE is distinguishable by E4.5 as an epithelial layer formed on the surface of the ICM exposed to the blastocoel. This segregation begins to take place at around E3.5 when individual cells of the ICM begin to display different gene expression patterns (Chazaud et al., 2006). The epiblast cells can be distinguished by expression of the pluripotency marker Nanog and the PrE cells by the markers Gata-4 and -6 (Fig1.1). By E4.5 their expression is mutually exclusive and the lineages are established.

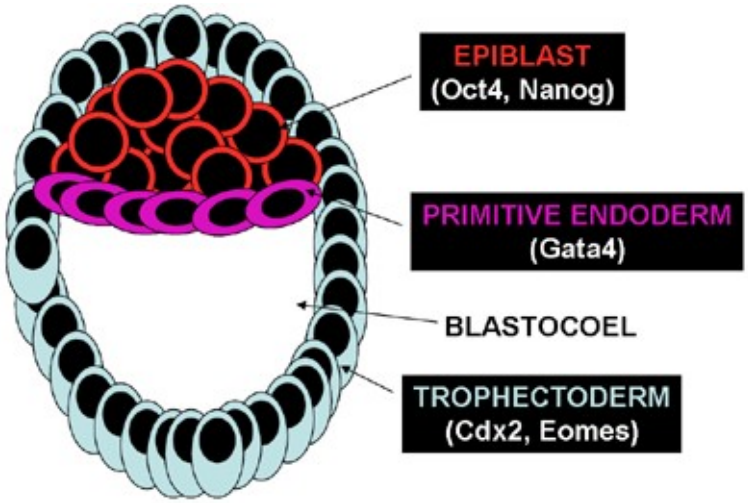


Fig1.1 Schematic representation of a blastocyst. The epiblast will give rise to all tissues of the embryo proper and is the origin of ES cells. It can be distinguished by expression of markers including Oct4 and Nanog. The primitive endoderm and trophectoderm give rise to extraembryonic tissues and can be identified by expression of Gata4 and Cdx2/Eomes respectively.

ES cells are normally derived from embryos harvested at developmental stage E3.5 (Evans and Kaufman, 1981; Martin, 1981) although they have been derived by culturing cells taken from the 8-cell embryo (Meissner and Jaenisch, 2006). Although the pluripotent cells of an E6.0 embryo have not committed to a particular germ layer (Lawson et al., 1991) they have lost the capacity to form ES cells indicating that there is a developmental 'window' during which it is possible to capture the ES cell state. The derivation of ES cells can be facilitated by inducing delayed implantation, known as diapause (Evans and Kaufman, 1981). Diapause is a natural phenomenon whereby pregnant mice still nursing young pups can delay the implantation of developing embryos for up to one month (Ozias and Weitlauf, 1971), effectively stalling their development. Diapause can be experimentally induced by tamoxifen injection or ovariectomy allowing researchers to isolate blastocysts arrested preimplantation.

The pluripotency of ES and EC cells can be tested by inducing differentiation *in vivo* or *in vitro*. Expanding lines from single cells generates clones that can be differentiated through embryoid body formation (Martin and Evans, 1975a) or teratocarcinoma formation following injection of cells subcutaneously (Martin,

1981). If cell types from all three germ layers are detected the cells from which they originated are described as pluripotent. The most stringent test of cultured pluripotent cells is their ability to contribute to chimaeras. Following injection into the developing embryo both EC cells (Brinster, 1974) and ES cells can contribute to tissues of the adult mouse. However, ES cells contribute with greater efficiency and do not display tissue biases as EC cells do. Furthermore, ES cells can contribute to the germ line (Bradley et al., 1984) whereas EC cells have never been known to do so because their aneuploidies prevent progression through meiosis.

1.2.1.1 The relationship between ES cells and the early embryo

ES cells are derived from the epiblast of the preimplantation embryo (Brook and Gardner, 1997). *In vivo* the pluripotent compartment exists only transiently. Thus, ES cells are defined as *in vitro* cell lines. Although ES cells are often used to model early embryonic development the relationship between ES cells and the cells of the developing embryo is not entirely clear. Do ES cells correspond to a particular developmental time point and does the process of derivation and subsequent *in vitro* culture change their properties? A key observation is that upon reintroduction to the embryo ES cells can give rise to all cell types of the adult mouse but never contribute to extraembryonic tissues (Gardner, 1985; Rossant et al., 2003). This suggests that ES cells correspond to epiblast cells at a stage beyond specification of the trophectoderm and primitive endoderm. However, ES cells can be induced to form trophoblast by down-regulation of Oct4 (Niwa et al., 2000) or forced expression of Cdx2 (Niwa et al., 2005). Formation of primitive endoderm occurs spontaneously under certain culture conditions and can be triggered by down-regulation of Nanog (Mitsui et al., 2003) or forced expression of Gata-4 or -6 (Fujikura et al., 2002). These differentiation events are considered illegitimate because they do not ordinarily occur *in vivo* and may represent a relaxation of developmental constraints associated with *in vitro* culture.

1.2.1.2 Lessons from epiblast stem cells

Recently, pluripotent cell lines were established from the post-implantation epiblast of the mouse embryo (EpiSCs, see below) (Brons et al., 2007; Tesar et al., 2007). The conditions used to establish and maintain these lines were identical to conditions optimised for the maintenance of hES cell lines based on the addition of activin and FGF2 to the media (Vallier et al., 2005). This, together with the report of rat EpiSCs (Brons et al., 2007), implies that there is cross-species conservation of a cell type that responds to these signals. Furthermore, transcriptional profiling of these lines revealed that hES cells were more similar to mEpiSCs than they were to mES cells (Tesar et al., 2007). This strongly suggests that hES cells also correspond to this later stage of development. Paradoxically, EpiSCs (Brons et al., 2007) and hES cells (Xu et al., 2002) can spontaneously differentiate into trophoblast cells. This developmental pathway is not expected to be open to cells at this later stage of development and may reflect properties acquired upon *in vitro* culture. hES cells are derived from blastocyst stage embryos and were initially derived under culture conditions very similar to those used to establish mES cells. Importantly, FGF2, which is now considered essential for hES cell culture, was not used in the first derivations (Thomson et al., 1998). This implies that the cells initially responding to *in vitro* culture correspond to a different developmental stage to that of the established lines. During derivation and/or subsequent culture the cells presumably progress to the later, FGF- and activin-responsive, developmental stage reflected by EpiSCs. It remains to be seen if the cells of the human pre-implantation epiblast share properties with their mouse counterparts and can be “captured” at this earlier stage. Anecdotal reports indicate that it is possible to convert ES cells to EpiSCs supporting the idea that development can proceed from pre- to post-implantation epiblast in culture. In contrast, EpiSCs cannot be converted to an ES phenotype.

1.2.2 Self-Renewal

Self-renewal is the division of a cell to produce two daughter cells, one or both of which are identical to the parental cell from which they arose. ES cells possess the

ability to self-renew without apparent limit and to give rise to all the cell types of the adult (Smith, 2001). These properties can be considered an *in vitro* phenomenon since the pluripotent cells of the embryo exist only transiently *in vivo*. The process of ES cell derivation is therefore the "capture" of a transient state. Maintenance of the pluripotent state *in vitro* is dependent on the expression of intracellular determinants and on extracellular signals (reviewed in (Chambers and Smith, 2004)). The intracellular determinants of ES cell identity can be detected in the pluripotent cells of the embryo where they perform essential roles in embryonic development (Nichols et al., 1998; Mitsui et al., 2003) whereas the role of extracellular signals in self-renewal may be solely an *in vitro* phenomenon. In order to sustain a self-renewing population of ES cells the culture environment must provide the nutrients and signals required to support cell division and to prevent apoptosis and must also inhibit the natural tendency of ES cells to differentiate. Variations on the culture environment exist and it is likely that different combinations of extracellular signals can support self-renewal. Fully defined conditions for the propagation of mES cells have been established (Ying et al., 2003a) and to a certain extent the signalling pathways involved have been elucidated (Niwa et al., 1998; Burdon et al., 1999b; Matsuda et al., 1999; Ying et al., 2003a). However, it has become clear that the findings in mES cells will not translate directly to hES cells (Thomson et al., 1998; Reubinoff et al., 2000; Daheron et al., 2004; Humphrey et al., 2004). Understanding the differences and similarities between mouse and human ES cells will help to define the requirements for hES cell derivation, *in vitro* culture and differentiation.

1.2.3 Intrinsic Determinants

The property of pluripotency is conferred by the expression of a set of transcription factors. Oct4, a Pou-domain transcription factor, was identified when its expression was observed in unfertilized oocytes, embryonic stem cells and primordial germ cells (Scholer et al., 1989). More recently, Nanog, a protein whose overexpression is capable of maintaining cytokine-independent self-renewal, was identified (Chambers et al., 2003; Mitsui et al., 2003). These proteins are the best known markers of pluripotency both *in vivo* and *in vitro* and their expression is common to all

pluripotent cell types that can be maintained *in vitro* and across those species from which ES cells have been derived.

1.2.3.1 Oct4 and Sox2

Oct4 is a member of a family of POU-domain transcription factors. It is expressed in the unfertilized egg and the early embryo and is down-regulated in the trophectoderm upon segregation from the inner cell mass (Pesce and Scholer, 2001). *Oct4* expression is maintained in the epiblast and segregated to migratory primordial germ cells (PGCs), persisting through genital ridge formation (Scholer et al., 1989).

Embryos lacking *Oct4* form outgrowths consisting entirely of trophectoderm-like cells when plated on gelatine (Nichols et al., 1998). This observation suggested a role for Oct4 in inhibition of differentiation into trophectoderm, supported by the observation that suppression of *Oct4* expression in mES cells results in 'dedifferentiation' of the cells into trophectoderm (Niwa et al., 2000). *Oct4* expression is continuously required for the maintenance of pluripotency but its expression is not sufficient. Removal of LIF results in differentiation even if Oct4 levels are artificially maintained by an inducible transgene. Furthermore, the level of *Oct4* expression is critical. Increasing expression to levels greater than 150% of wild-type levels results in differentiation into mesoderm/endodermal lineages while a reduction to less than 50% of wild-type levels results in dedifferentiation into trophectoderm (Niwa et al., 2000).

Exactly how Oct4 controls cell fate in ES cells and *in vivo* remains unclear. The sensitivity of ES cells to levels of Oct4 suggests that interaction with other transcription factors and the relative level of expression of these is critical to Oct4 function (Niwa et al., 2000). Niwa modelled self-renewal around Oct4 and its differing effects on target genes. Target genes were divided into three groups based on their response to Oct4 (activation or repression) and activated targets could be divided again based on whether or not they were repressed by high levels of Oct4, a phenomenon known as 'squelching' (Scholer et al., 1991). In such a model

interacting partners of Oct4 are key. Sox2 is known to be required for expression of Oct4 target genes in ES cells. The best characterised Oct4 target is *Fgf4*. Sox2 was identified as the factor required to activate *Fgf4* expression together with Oct4 (Yuan et al., 1995). Oct4 and Sox2 form a ternary complex by binding closely-spaced enhancer elements in the *Fgf4* promoter. The spacing of the Oct4 and Sox2-binding sites is critical for promoter activity, seemingly because it facilitates Oct4 and Sox2 interaction (Ambrosetti et al., 1997). Another gene with ES cell specific expression, *Utf1*, is similarly regulated by Oct4 and Sox2 (Nishimoto et al., 1999). More recently it has been shown that Oct4 and Sox2 reciprocally regulate their own expression (Tomioka et al., 2002; Chew et al., 2005). Both *Sox2* and *Pou5f1* promoter regions contain oct-sox elements that have been shown to bind Oct4 and Sox2. These findings, together with the demonstration that expression of the key pluripotency gene, *Nanog* (see below), is regulated by Oct4 and Sox2 (Rodda et al., 2005), imply that the Oct4-Sox2 interaction is critical for maintenance of ES cells. Indeed, loss of *Sox2* expression results in differentiation in a manner virtually identical to that seen upon suppression of *Oct4* supporting the idea that the key role of Sox2 in ES cells is in the activation of Oct-Sox target genes. Surprisingly however loss of Sox2 could be rescued by the forced expression of *Oct4*. Oct-Sox target genes were activated in a Sox2-independent manner, probably as a result of redundancy with other Sox family members, and pluripotency could be maintained (Masui et al., 2007). This work shows that the key contribution of Sox2 is in fact to maintain levels of Oct4 through regulation of transcription factors that affect *Oct4* expression.

Some light has been shed on the role of Oct4 through studies of its interactions with Cdx2, a marker of trophectoderm. It was found that overexpression of *Cdx2* leads to trophectoderm differentiation in a manner virtually indistinguishable from that observed when *Oct4* is extinguished (Niwa et al., 2005). Interestingly, differentiation proceeded in *Cdx2*-overexpressing cells even when *Oct4* expression was artificially maintained from a transgene demonstrating that loss of *Oct4* expression is not required for differentiation. Cdx2 and Oct4 are both capable of positively regulating their own transcription and they reciprocally inhibit one-another's activation. The indication that Cdx2 and Oct4 physically interact and that this apparently neutralizes

their transcriptional activation functions suggests that the battle between these two transcription factors is important in the first lineage segregation in the embryo (Smith, 2005). Indeed, both proteins can be detected in all cells of the 8-cell morula before becoming progressively restricted to the TE and ICM respectively (Niwa et al., 2005). Cdx2 expression is first observed just after compaction, at which point most cells express Cdx2 (Dietrich and Hiiragi, 2007). As the embryo becomes large enough to accommodate inside (inside cells are those completely surrounded by other cells) and outside cells Cdx2 expression shows a strong bias for outside cells. Interestingly, upregulation of Cdx2 initially shows no correlation with Oct4 levels with Oct4 expression remaining high in all blastomeres of the embryo until the late blastocyst stage when it becomes down-regulated in the Cdx2-high trophectoderm (Dietrich and Hiiragi, 2007). The fact that Cdx2 expression is established and maintained in the presence of Oct4 and that this does not lead to immediate down-regulation of Oct4 suggests that there are other regulatory mechanisms at play in the segregation of ICM and TE. Reciprocal regulation of Oct4 and Cdx2 may serve to reinforce rather than establish gene expression patterns in the preimplantation embryo.

1.2.3.2 Nanog

Nanog was identified independently by two groups through a functional cDNA screen for genes conferring LIF-independent self-renewal (Chambers et al., 2003) and through differential expression analysis (Mitsui et al., 2003). Nanog is a divergent homeodomain protein expressed first in the morula with high levels persisting in the early blastocyst and declining prior to implantation. Following implantation *Nanog* expression is restricted to a subset of epiblast cells and upon entry to the primitive streak is rapidly down-regulated. *Nanog* can still be detected in PGCs during migration to and colonisation of genital ridges but is subsequently down-regulated in the germ cell lineage.

Nanog overexpression can drive cytokine-independent self-renewal of ES cells. This ability requires the continued expression of *Oct4*. Differentiation caused by the elimination of *Oct4* expression can be rescued by transfection with an *Oct4*-expressing but not a *Nanog*-expressing plasmid (Chambers et al., 2003). This result also implies that Nanog can direct *Oct4* expression either directly or indirectly. Nanog does not function through activation of the JAK/STAT pathway (see below for a discussion of JAK/STAT signalling) as *Nanog*-overexpressing cells continue to self-renew even when SOCS3, a potent inhibitor of the JAK/STAT pathway, is overexpressed. However, self-renewal occurred with greater efficiency when *Nanog*-overexpressing cells were also stimulated with LIF, implying that these pathways function in parallel (Chambers et al., 2003).

Nanog-null ES cells, generated by serial targeting of the *Nanog* locus to replace the homeodomain by homologous recombination, acquired a parietal endoderm-like morphology and expressed markers of parietal and visceral endoderm. Expression of *Oct4* and *Rex1* was reduced but not eliminated when the cells were maintained on STO feeders. In the absence of feeders their expression was completely eliminated while expression of Gata6 was further induced (Mitsui et al., 2003). Recently, however, these results have been brought into question by the finding that deletion of *Nanog* from cells expressing an inducible form of *Nanog*, and subsequent suppression of *Nanog* expression, does not result in inevitable differentiation. These null cells can be passaged and grown long term in culture while retaining the ability to form tissues of all three germ-layers (Chambers et al., 2007). These findings suggest that Nanog is required for the establishment of, but not maintenance of, a pluripotent phenotype. This would predict that the requirement for Nanog in the developing embryo is absolute, and indeed this seems to be the case. At 3.5dpc *Nanog*-null blastocysts were indistinguishable from normal embryos. By E5.5 embryos consisted entirely of disorganized extraembryonic tissues with no discernible epiblast or extraembryonic ectoderm. Explanted ICMs failed to proliferate and differentiated completely into parietal endoderm-like cells within 4 days. The fact that no trophectoderm was observed implies that Nanog is required at a stage beyond the initial requirement for Oct4 (Mitsui et al., 2003).

It has been suggested that Nanog might function, at least in part, through inhibition of Gata factors (Mitsui et al., 2003; Chambers and Smith, 2004). Forced expression of *Gata4* or *Gata6* in ES cells is sufficient to drive differentiation into extraembryonic endoderm (Fujikura et al., 2002) while *Nanog*-null cells spontaneously differentiate into extraembryonic lineages expressing high levels of *Gata-4* and *-6* (Mitsui et al., 2003). In both cases the expression profile is very similar. A putative Nanog-binding site has also been identified in the enhancer region of *Gata6* but has yet to be demonstrated to bind Nanog (Mitsui et al., 2003). Given that *Nanog* overexpression prevents lineage commitment of ES cells, not just to endoderm, but also to cell types of the ectoderm and mesoderm germ layers it is unlikely that Nanog functions solely to inhibit Gata transcription factors.

Recently Nanog has been demonstrated to interact with Sall4 (Wu et al., 2006). Nanog and Sall4 appear to bind together to their own promoter regions and to positively regulate transcription forming a feed-forward loop. Sall4 was found to co-occupy the promoter regions of many Nanog targets implying that there may be functional significance to the interaction. A separate study showed that Sall4 regulated *Oct4* expression and that depletion of Sall4 in the embryo resulted in *Cdx2* expression in the ICM, a phenotype consistent with loss of Oct4 (Zhang et al., 2006). However, *Sall4*-null ES cells have been derived and were reported to have a proliferation defect but no increase in differentiation (Sakaki-Yumoto et al., 2006). It has been suggested that the discrepancy between these findings and those of Zhang *et al* result from a failure to examine feeder-independent self-renewal (Zhang et al., 2006) but the *in vivo* phenotype of *Sall4*-null blastocysts indicates that they remain capable of establishing the pluripotent compartment (Sakaki-Yumoto et al., 2006). The failure of these studies to agree on the role of Sall4 in ES cells and the early embryo indicates that further experiments will be required before we can be confident that Sall4 is a significant player in the regulation of pluripotency.

Nanog has been implicated in the establishment of a pluripotent epigenome. *Nanog* expression was found to enhance the efficiency of reprogramming of somatic cell

types to pluripotent cell types by cell fusion (Silva et al., 2006). This, together with the observation that *Nanog*-null cells, while retaining the ability to contribute to chimeras, cannot contribute to the germ-line suggests that Nanog may play a role in establishing the pluripotent state during normal development and *in vitro* during reprogramming. The observation that ES cells which have little or no Nanog can re-express Nanog and remain pluripotent but that cells lacking Nanog are more prone to differentiation suggests that in the artificial context of ES cell *in vitro* culture Nanog supports self-renewal by 'guarding' or resetting the pluripotent state of the cells (Chambers et al., 2007). This is consistent with the reduction in spontaneous differentiation observed upon forced expression of *Nanog* (Chambers et al., 2003). In light of this, it was surprising to learn that *Nanog* was not one of four factors which, when expressed together, are capable of reprogramming somatic cells to pluripotency (Takahashi and Yamanaka, 2006). However, selection for reactivation of *Nanog* expression, as opposed to *Fbx15*, another gene associated with pluripotency, enhanced the efficiency of reprogramming to pluripotency (Okita et al., 2007) suggesting there may be a role for the endogenous *Nanog* gene (see below for a more in depth discussion of reprogramming).

1.2.3.3 Pluripotency Network

As described above the transcription factors Oct4, Sox2 and Nanog play critical roles in the establishment of the pluripotent epiblast *in vivo* and in the maintenance of pluripotency *in vitro*. As a result a great deal of time and effort has been invested to attempt to describe their mechanism of action.

Microarray and chromatin immunoprecipitation (ChIP) experiments have been performed to identify transcriptional targets of the three transcription factors. Experiments designed to identify promoter regions bound by these transcription factors on a genome wide scale revealed that a high proportion of promoters occupied by one TF would also be occupied by the others (Boyer et al., 2005; Loh et al., 2006). This suggests that they cooperate in their regulation of target genes. Comparison of promoter occupation with global expression analyses revealed that

the TFs occupied the promoters of both active and inactive genes (Boyer et al., 2005) and both Oct4 and Nanog appeared capable of positive and negative regulation of transcription (Loh et al., 2006). Although it is not known how Oct4, Sox2 and Nanog might mediate both positive and negative effects on transcription it is notable that the active genes included many implicated in pluripotency and inactive genes were enriched for developmental regulators (Boyer et al., 2005). In one study the authors find that RNAi-mediated knock down of two positively regulated targets of both Nanog and Oct4 (*Rif1* and *Esrrb*) results in differentiation (Loh et al., 2006), suggesting that the cooperation of these TFs is of functional significance for the regulation of pluripotency. These global studies are flawed in that they clearly reveal the occupancy of many sites that have no functional relevance and because changes in gene expression levels upon differentiation are interpreted as direct targets failing to acknowledge the possibility that many changes are simply an indirect consequence of differentiation. However, they propose a framework for the regulation of a pluripotent epigenome: the key pluripotency-associated TFs, by positively regulating their own and each other's expression, form a stable feed-forward loop (Fig1.2). By silencing genes associated with specification of differentiated cell types they prevent differentiation, allowing the pluripotent compartment to expand *in vivo* and ES cells to be propagated indefinitely *in vitro*. This model is supported by a study of protein interactions which suggests that the key TFs, Oct4 and Nanog, are part of multi-protein complexes (Wang et al., 2006). A strong bias was observed among the interacting proteins identified for knockout phenotypes (where studied) in the early embryo and ES-cell associated expression patterns suggesting a functional role in pluripotent cells. The interacting proteins identified also tended to be part of known cofactor pathways involved in transcriptional repression. For example, Nanog can be linked to histone deacetylase (HDAC) and NuRD complexes through its association with Sall1/4 and Nac1 (Wang et al., 2006). This is consistent with the idea that pluripotency-associated TFs act to silence expression of genes associated with lineage commitment.

Fig1.2. Nanog, Oct4 and Sox2 cooperate to block lineage commitment. Nanog, Oct4 and Sox2 positively regulate one-another's transcription forming a stable feed-forward loop. These transcription factors co-regulate many genes and block lineage commitment although details of their mechanism of action remain largely unknown.

Recent findings from in depth studies of single TFs may call for a re-evaluation of this network. Nanog is dispensable for the maintenance of ES cells (Chambers et al., 2007) and the critical contribution of Sox2 appears to be the maintenance of *Oct4* expression (Masui et al., 2007). Only the expression of *Oct4* remains an absolute requirement for ES cell propagation although the requirement for Nanog and Sox2 remains in the developing embryo.

1.2.4 Epigenetic Regulation of Pluripotency

ES cells possess highly dynamic, decondensed chromatin (Meshorer et al., 2006) as evidenced by the early replication of unexpressed genes during S-phase (Azuara et al., 2006); early replication is normally associated with actively transcribed loci. ES cells were also observed to have an interesting pattern of chromatin modifications. Large regions of the repressive histone mark, trimethyl histone 3 lysine 27 (H3K27me3), were found to harbour smaller regions of the active mark H3K4me3 and were termed 'bivalent domains' (Azuara et al., 2006; Bernstein et al., 2006). The coexistence of these marks is provocative, suggesting that the chromatin is being maintained as accessible but the genes are being kept silent by the repressive marks. Furthermore, the bivalent domains were enriched in regions encoding transcription factors that were not expressed or expressed at low levels. Methylation of H3K27 is mediated by Polycomb Repressive Complexes (PRC) -1 and -2. To investigate a potential role for these complexes in pluripotency their binding to genomic DNA was mapped by chromatin immunoprecipetation (ChIP) using antibodies against components of both complexes and interrogating microarrays representing promoter

sequences with the isolated genomic DNA (Boyer et al., 2006) (a technique known as ChIP on Chip). A group of promoters binding both PRC-1 and -2 were identified and found to be enriched for TFs with known roles in development. These genes also showed preferential upregulation upon differentiation implying a functional significance of the PRCs in silencing genes that are poised to be expressed. Furthermore, ES cells lacking a component of the PRC2 complex, Eed, and thus lacking H3K27me3 marks (Montgomery et al., 2005) showed loss of repression of the genes identified as bound by the PRC complexes. Together with the observation that in hES cell promoters bound by the polycomb group protein Suz12 were enriched for binding sites for Oct4, Sox2 and Nanog (Lee et al., 2006) has lead to the hypothesis that PRCs may somehow be controlled by the core TFs of pluripotency. The significance of these findings must not be overstated however. Studies of global histone marks in hES cells reveals that although they too have bivalent domains, the co-existence of H3K27me3 and H3K4me3 is the rule rather than the exception (Pan et al., 2007; Zhao et al., 2007). The majority of promoters with the H3K27me3 mark also had the H3K4me3 mark. Furthermore, pluripotency-associated genes were observed to acquire H3K4me3 during differentiation (Pan et al., 2007), becoming bivalent as pluripotency was lost. Bivalent marks were associated with silent genes and were not a unique feature of pluripotent cells (Pan et al., 2007). Finally, when genes marked by different combinations of the two histone marks were examined for changes in expression during differentiation all combinations revealed a proportion of genes that responded rapidly (Pan et al., 2007). This also casts doubt on the idea that bivalent marks are associated with rapidly-responding genes. Finally, the fact that ES cells can be maintained in the absence of functional PRCs argues against an essential role in the maintenance of pluripotency (Montgomery et al., 2005; Schoeftner et al., 2006; Pasini et al., 2007).

A recent study has demonstrated a link between the core TFs of pluripotency and epigenetic regulators (Loh et al., 2007). Oct4 regulates the transcription of the H3K9 demethylases *Jmjd1a* and *Jmjd2c*. *Jmjd1a* demethylates H3K9me2 while *Jmjd2c* demethylates H3K9me3. RNAi mediated knock-down of either results in differentiation. The examination of gene expression changes following knock-down

and subsequent ChIP experiments revealed *Tcl1* as a JMJD1A target while *Nanog* is a JMJD2C target. The increased H3K9me2 levels at the *Tcl1* promoter reduce Oct4 binding and hence *Tcl1* expression. Differentiation as a result of knock-down could be rescued by simultaneous expression of *Tcl1* or stable expression of *Nanog* for *Jmjd1a* and *Jmjd2c* respectively. These experiments link the activity of Oct4 targets to modulation of the chromatin to facilitate expression of pluripotency-associated TFs (Niwa, 2007a). However, these experiments raise questions unaddressed by the authors: down-regulation of histone demethylases is predicted to result in increased methylation of H3K9 and a subsequent reduction in transcription but the up-regulation of some genes upon down-regulation of *Jmjd1a* or *Jmjd2c* implies off-target or secondary effects; it is surprising that loss of epigenetic modifiers and the resulting changes in expression of hundreds of genes can be rescued by the expression of a single transcription factor with no known chromatin-modifying activity; knock-down of *Jmjd1a* can be rescued by *Tcl1* but not *Nanog* expression even though *Nanog* has previously been shown to rescue depletion of *Tcl1* in ES cells (Ivanova et al., 2006).

While the study of the epigenetic status of ES cells has recently received a great deal of attention it is worth considering a few points. Firstly, the pluripotent phenotype is dominant and capable of imposing itself on a somatic genome following cell fusion (Tada et al., 2001) and recently the generation of pluripotent cells from somatic cells has been achieved by the expression of 4 TFs (Takahashi and Yamanaka, 2006). This demonstrates that a terminally differentiated genome can be forced to reprogram by exposure to factors associated with pluripotency and that TFs alone are sufficient to direct the epigenetic changes required for reprogramming. Secondly, while in depth studies have clearly demonstrated the critical role of several TFs in the establishment and/or maintenance of pluripotency, ES cells have been shown to tolerate quite severe disruptions to their epigenetic machinery while retaining the characteristics of pluripotency (Montgomery et al., 2005; Pasini et al., 2007). *In vivo*, disruption of the epigenetic machinery results in severe developmental abnormalities but in the majority of cases lethality occurs after the pluripotent compartment of the embryo has been established and in most cases mutant ES cells have been established

(reviewed in (Surani et al., 2007)). It is likely that the TFs of the pluripotency network employ chromatin modifying complexes to set up and maintain their transcriptional program but it is the action of the core TFs that directs the epigenetic modifications and not an epigenetic program that dictates expression of the TFs.

1.2.5 Pluripotent Cell Types

ES cells are derived from the preimplantation embryo. Investigations into the nature of pluripotency have lead researchers to attempt the derivation of pluripotent cells from alternative sources. As well as furthering our knowledge of developmental biology and epigenetic reprogramming it is hoped that it will become possible to generate pluripotent cell types without use of human embryos. Cells with pluripotent properties have been derived from the germ cell lineage, from post-implantation embryos (Brons et al., 2007; Tesar et al., 2007) and by reprogramming of somatic cells (Takahashi and Yamanaka, 2006).

1.2.5.1 Embryonic Germ Cells and Spermatogonial Stem Cells

Pluripotent stem cells with properties similar to ES cells have been generated from various stages of germ cell development. The germ lineage is the only place apart from the pluripotent cells of the pre- and peri-implantation embryo where genes associated with pluripotency are expressed. *In vitro* cell lines can be established from Primordial germ cells (PGCs) isolated from the E8.5-12.5 embryo and maintained long-term in culture on a feeder layer in media supplemented with stem cell factor (SCF, also known as steel factor), LIF and basic fibroblast growth factor (bFGF) (Matsui et al., 1992; Resnick et al., 1992). These cells are known as embryonic germ (EG) cells and they express markers of pluripotency, are capable of *in vitro* differentiation into cells representative of the three germ layers, form teratomas when injected subcutaneously, and contribute extensively to chimaeras, including the germ line (Labosky et al., 1994), following blastocyst injection (Matsui et al., 1992). Although the growth factors were absolutely required for establishment of the

primary cultures, established cell lines could be maintained in ES cell growth conditions (Matsui et al., 1992). PGCs cannot contribute to chimaeras when transplanted directly to the embryo implying that the establishment of EG cells is a reprogramming event.

Spermatogonial stem cells (SSCs) have been isolated from neonatal mouse testis. Dissociated testis plated in glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), bFGF, and LIF give rise to cell lines that express markers of spermatogonia and can restore fertility to infertile mice (Kanatsu-Shinohara et al., 2003). The authors of this work noticed that ES-like cells appeared in the cultures, showed that these cells gained expression of ES cell markers SSEA1 and *Nanog*, which are not expressed in SSCs, and that they could be propagated in ES cell culture conditions (Kanatsu-Shinohara et al., 2004). These cells were termed multipotent germ-line stem (mGS) cells and were phenotypically indistinguishable from ES cells with the exception of genomic imprinting. mGS cells could not be established by direct culture of testis in ES cell conditions and the establishment of SSCs is absolutely dependent on the presence of GDNF. mGS cells differentiated efficiently *in vitro*, in teratomas and contributed to chimaeras including the germ line. It has since been shown that ES-like cells can be established from the testis of adult mice by isolating cells expressing GFP under the control of the *Stra8* promoter (Guan et al., 2006) which marks spermatogonia. These cells could be expanded when plated directly in ES cell culture conditions without the requirement for prior culture in GDNF-containing culture conditions. The cells were demonstrated to be pluripotent by contribution to germ-line chimaeras and were termed multipotent adult germline stem cells (maGSCs). The apparent direct conversion of SSCs to pluripotent maGSCs implies that SSCs of the adult testis are themselves pluripotent or that they respond rapidly to the culture conditions to become ES-like cells. It is not clear why this group succeeded in establishing ES-like cells from adult testis and without the requirement for GDNF in primary cultures but the finding is extremely exciting because it promises that pluripotent stem cells will be derived without the need for embryos and that autologous stem cells might become available (Kanatsu-Shinohara and Shinohara, 2006).

In the case of both EG and GS cells the contribution to chimaeras is proof of their pluripotency and distinguishes them from Epiblast Stem Cells (see below). In both cases the cells from which they are derived are not thought to be pluripotent and their derivation might therefore be considered a form of reprogramming. It is likely that cells of the germ line are particularly susceptible to reprogramming to pluripotency because they already express key TFs associated with a pluripotent phenotype. They are also thought to be the cells responsible for initiating spontaneous teratocarcinomas, the tumours that arise when pluripotent cells are injected into a permissive site, suggesting that they possess or are predisposed to develop the properties of self-renewal required for *in vitro* propagation.

1.2.5.2 Reprogramming and Induced Pluripotent Stem Cells

Reprogramming refers to the process of altering the epigenetic program of a cell to change its developmental potential. Converting a somatic cell to a pluripotent cell has long been the goal of researchers. Cloning by somatic cell nuclear transfer (SCNT) requires that the genome of a terminally differentiated cell is reprogrammed to acquire the potential to produce all cell types of the developing organism ie. to totipotency. Reprogramming requires that the epigenetic marks are wiped from the genome to allow reexpression of silenced genes. If somatic cells could be induced to acquire pluripotency it would provide an alternative source to embryos and allow the production of patient-specific stem cells. Reprogramming has been achieved by transferring the nucleus of a somatic cell to an enucleated egg (SCNT) and by fusing somatic cells with pluripotent cells. SCNT still requires the use of oocytes and is highly inefficient while cell fusion results in the production of tetraploid cells which could not be used in cell replacement therapy because of potential problems including tumorigenesis of tetraploid cells and the acquisition of non-autologous chromosomes from the pluripotent donor genome (Ambrosi and Rasmussen, 2005). Recently however it has been demonstrated that somatic cells can be reprogrammed by the introduction of 4 genes encoding the TFs *cMyc, Oct4, Sox2* and *Klf4* (Takahashi and Yamanaka, 2006). There is no need to use oocytes, the resulting cells

are diploid and the process has the potential to be used to generate patient-specific pluripotent cells. The cells produced by this process are termed induced pluripotent stem (iPS) cells.

It has now been demonstrated that both mouse (Takahashi and Yamanaka, 2006; Wernig et al., 2007) and human (Takahashi et al., 2007; Yu et al., 2007) iPS cells can be derived. Thus, it is now possible to take cells from an adult human, convert them to pluripotent cell lines with apparently limitless capacity for expansion and use these to generate clinically relevant cell types. In a proof-of-principle experiment, carried out in mice, iPS cells were generated from skin cells of mice expressing the human sickle haemoglobin allele. The genetic defect was corrected in the iPS cells and the resulting cells used to generate haematopoietic progenitors which rescued the anaemia when transplanted (Hanna et al., 2007).

iPS cells were first generated from mouse embryonic fibroblasts (MEFs). The 4 TFs were introduced by retroviral transfection and ES-like cells were selected for by selecting for drug resistance under the control of the promoter of the pluripotency-associated gene, *Fbx15* (Takahashi and Yamanaka, 2006). ES-like cells were isolated and demonstrated to be very similar to ES cells, possessing pluripotency as assessed by *in vitro* differentiation and teratoma formation. However, the cells could not contribute to the germ-line when chimeras were formed by blastocyst injection. Germ-line transmission was later achieved by selecting for the expression of *Nanog* (Okita et al., 2007) or *Oct4* (Wernig et al., 2007). This probably reflects a requirement for activation of the endogenous *Oct4* and *Nanog* transgenes before true pluripotency is established (Silva and Smith, 2008).

As yet it is not known how each of the 4 factors acts to convert the somatic cell genome to pluripotency. Oct4 and Sox2 most likely act to promote expression of the endogenous *Oct4* and *Sox2* genes (Tomioka et al., 2002; Okumura-Nakanishi et al., 2005) as well as *Nanog* (Rodda et al., 2005). Once active this pluripotency network is expected to be self-promoting and will also begin to influence the activity of target genes whose expression or silencing is critical to the establishment of pluripotency.

cMyc and *Klf4* may act as oncogenes to transform somatic cells to the highly proliferative, immortal phenotype of stem cells (Jaenisch and Young, 2008). It has also been suggested that cMyc can influence the structure of chromatin and may promote a more open conformation which is more amenable to the epigenetic conversion to pluripotency (Jaenisch and Young, 2008). *Klf4* is capable of influencing the activity of Oct4/Sox2 target genes (Nakatake et al., 2006) and may therefore act in a more direct manner to influence the expression of key target genes. Both *cMyc* and *Klf4* are dispensable for the generation of iPS cells (Yu et al., 2007; Nakagawa et al., 2008) but the efficiency and speed of conversion is greatly enhanced by their inclusion. This is consistent with the view that the key factor is the master regulator of pluripotency, Oct4 (Jaenisch and Young, 2008).

However, several major problems must be overcome before iPS cells can be used in treatments for humans. The initial conversion is currently driven by genomic integration of the transgenes encoding the TFs. Ideally integration would be avoided to prevent integrative mutagenesis. Furthermore, both *cMyc* and *Klf4* are known oncogenes. Although the retroviral insertions appear to be silenced in iPS cells they could become activated upon differentiation. Chimaeric mice generated from iPS cells have been observed to form tumours, thought to result from reactivation of the *cMyc* transgene (Okita et al., 2007). To avoid these problems it is hoped that it will be possible to generate iPS cells without integrating the transgenes. This may be possible by episomal expression of the TFs but the length of time and the high levels of transgene expression required may mean that episomal transfection will be ineffective. An alternative would be to use recombinant proteins. It has been demonstrated that fusion of a TAT peptide to the protein of interest enhances cellular uptake (Peitz et al., 2002) which would clearly be necessary for recombinant TFs to work effectively.

1.2.5.3 Epiblast Stem Cells

Pluripotent cell lines have recently been derived from the post-implantation mouse and rat embryos (Brons et al., 2007; Tesar et al., 2007). They are established by

dissecting the epiblast of the E5.5-6.5 mouse embryo (E7.5 for the rat) and plating into conditions optimized for the growth of hES cells. They are termed epiblast stem cells (EpiSCs) to reflect their tissue of origin. The cells can be grown without apparent limit in culture and can be cloned, albeit with low efficiency. Their pluripotency has been established through *in vitro* differentiation and observation of multi-lineage differentiation in teratomas following injection into permissive sites in immuno-compromised mice. ES cells cannot be maintained in EpiSC conditions and explanted ICMs differentiate under these conditions. Similarly, EpiSCs cannot be maintained in ES cell conditions and isolated epiblasts cannot be expanded. This indicates that these are distinct cell types and this is confirmed by marker analysis. EpiSCs express genes normally expressed by the pre-gastrulation epiblast that are not detected in ES cells, for example *Fgf5*, and lack expression of some ES cell-associated genes such as *Rex1*. EpiSCs are similar in this regard to the previously described early primitive ectoderm-like (EPL) cells which are induced and maintained by an undefined activity in media conditioned by HepG2 cells (Rathjen et al., 1999). However, EPL cells can be reverted back to ES cells when the conditioned media is removed and LIF is present.

Contribution to chimeric animals following morula aggregation or blastocyst injection is a stringent test of pluripotency and of the ability of *in vitro*-cultured cells to re-enter their normal developmental program. ES cells contribute efficiently to all tissues including the germ line. The post-implantation epiblast is considered pluripotent but EpiSCs could not contribute to chimeras (Brons et al., 2007; Tesar et al., 2007). This could simply be due to incompatibility between the EpiSCs and the cells of the ICM but it has significant implications for these cells. Firstly, if they do not contribute to chimeras their use in the generation of transgenic animals would not be possible which is disappointing given that rat EpiSCs have apparently been established. Secondly, these cells cannot formally be considered truly pluripotent. Although Tesar *et al* report induction of the PGC-specific genes *Blimp1* and *Stella* in response to BMP4 they have not been shown to form functional gametes.

The most significant implication of the work on EpiSCs is a possible solution to the conundrum of the relationship between mouse and human ES cells. Although human ES cells were established from embryos at a similar developmental stage to those used in mES cell derivation and under conditions known to support mES cells they are phenotypically different. They share expression of the core transcriptional machinery of ES cells but differ in cell surface marker expression and in their appearance and behaviour. hES cells grow as large flattened colonies as opposed to the rounded up morphology exhibited by mES cells and do not tolerate well dissociation to single cells as mES cells do. The finding that mEpiSCs are more similar in their expression profile and in their pattern of Oct4 binding sites to hES cells than they are to mES cells suggests that hES cells correspond to a later developmental stage than previously assumed. The fact that they are derived from preimplantation embryos does not exclude this possibility since 'development' can continue *in vitro* and the stem cells derived may correspond to a different developmental stage to the embryo from which they came. If this is indeed the case then the same questions of whether they are truly pluripotent will apply as this can never be tested through production of human chimeras. The intriguing possibility is that a hES cell that corresponds to mES cells has been 'missed' and that this might still be isolated through the development of alternative culture conditions or alternative methods of derivation.

1.3 Extracellular Signals

Initially, mES cells were derived and cultured on a layer of mitotically inactivated fibroblasts in serum-containing medium (Evans and Kaufman, 1981; Martin, 1981). The fibroblasts were thought to provide trophic factors that support self-renewal and were hence described as feeders. This was confirmed by the observation that feeders could be replaced by the addition of medium conditioned by buffalo rat liver cells (Smith and Hooper, 1983). Subsequent fractionation of this conditioned medium identified leukaemia inhibitory factor (LIF) as the active component (Smith et al., 1988; Williams et al., 1988). Addition of recombinant LIF to serum-containing medium is sufficient to support self-renewal. In 2003 it was demonstrated that serum

could be replaced by the addition of recombinant BMP4 and for the first time ES cells could be grown in fully defined conditions (Ying et al., 2003a).

The apparent requirement for exogenous signals in the culture of ES cells has meant that a great deal of effort has gone into identifying signalling pathways that modulate self-renewal and the investigation of their downstream targets. However, the requirement for a particular signal may be context-dependent. For example, BMP4 normally induces differentiation of ES cells and only behaves as a self-renewal signal in the presence of LIF. Therefore any given signalling pathway might promote self-renewal only when other particular pathways are active/inactive. It is also true that signalling pathways affecting ES cells may not serve the same function in the developing embryo and researchers must be cautious when interpreting their data in the context of development. It seems logical to look for a link between the core TFs of pluripotency and the signalling pathways that promote self-renewal but there are few, if any reports clearly linking the signalling pathways with direct regulation of the TFs.

1.3.1 LIF and BMP Signalling

LIF is a member of the IL6 family of cytokines that signal through a common transmembrane receptor, gp130 (Boulton et al., 1994). Signalling mediated by gp130 homodimers (Yoshida et al., 1994) or by heterodimers consisting of gp130 and a related receptor such as the LIF receptor is capable of directing self-renewal in the presence of serum. Following ligand-induced receptor dimerization there are two main signalling pathways that become activated (Fig1.3). The intracellular domain of gp130 recruits tyrosine kinases such as the Janus Kinases (JAKs) (Boulton et al., 1994). JAKs phosphorylate tyrosine residues on the intracellular domain of gp130, creating binding sites for STAT3. Receptor-bound STAT3 is phosphorylated on Tyr705 by JAKs before dimerizing and translocating to the nucleus where it acts to modulate transcription of target genes. STAT3 has been reported to have activity independent of Tyr705 phosphorylation through phosphorylation on Ser727. Ser727 was observed to become phosphorylated in NS cells in response to Notch ligands and to enhance colony formation implying a role in cell survival (Androutsellis-

Theotokis et al., 2006). Shp2 binds a distinct phosphotyrosine residue on gp130. Signalling downstream of Shp2 mediates activation of the MAP kinases ERK-1 and -2 (Burdon et al., 1999a). A well characterised STAT3 transcriptional target is *Socs3* (Auernhammer et al., 1999). SOCS3 mediates a negative feedback loop by binding to the gp130 receptor and preventing activation of downstream signalling pathways. Forced expression of *Socs3* causes differentiation of ES cells even in the presence of LIF.

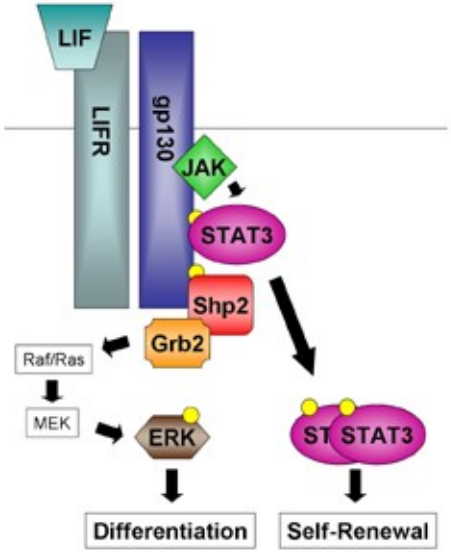


Fig1.3. LIF signalling. LIF binds to its receptor and induces dimerisation with gp130. Janus Kinases (JAKs) constitutively associated with gp130 become activated and phosphorylate Tyr residues on the receptor creating docking sites for SH2 domain-containing proteins including STAT3 and Shp2. Receptor-bound STAT3 is phosphorylated by JAKs, dimerizes, translocates to the nucleus where it activates transcription of target genes presumed to promote self-renewal. Recruitment of Shp2 leads to activation of ERK1/2 which promotes differentiation.

Activation of STAT3 was demonstrated to be the critical component of gp130 signalling in directing self-renewal. Selective elimination of tyrosine residues from the intracellular domain of gp130 demonstrated that STAT3 binding sites were required for self-renewal and that a chimeric receptor with only STAT3-binding tyrosines, that did not activate ERK1/2, was capable of supporting self-renewal (Niwa et al., 1998). Furthermore, a STAT3-Oestrogen Receptor (ER) fusion was capable of supporting self-renewal in the absence of exogenous LIF when activated by the addition of 4-hydroxy tamoxifen (4-HT) (Matsuda et al., 1999).

STAT3 homodimers function as TFs and it is supposed that transcriptional targets of STAT3 mediate self-renewal. One group identified *cMyc* as a target of STAT3 and went on to claim that constitutive expression of cMyc rendered ES cells LIF-independent (Cartwright et al., 2005b). The group propose that cMyc functions by

blocking differentiation but it is not clear how this is achieved nor is it clear if the effect remains dependent on the presence of serum in the culture medium. *Klf4* has also been shown to be a downstream target of LIF. Its overexpression in ES cells lead to decreased differentiation in EBs and enhanced secondary EB formation suggesting that it promotes self-renewal (Li et al., 2005). The recent identification of *cMyc* and *Klf4* as two of four factors capable of reprogramming somatic cells to pluripotency (Takahashi and Yamanaka, 2006) adds significance to their proposed roles in the self-renewal of ES cells but their mechanisms of action in either reprogramming or self-renewal remain to be clearly defined.

Embryos lacking *gp130* can develop normally beyond the establishment of the pluripotent epiblast (Yoshida et al., 1996). It is therefore unclear if gp130-STAT3 signalling plays any role *in vivo* analogous to the promotion of self-renewal seen *in vitro*. When *gp130-/-* embryos are induced to enter diapause and to arrest at the late blastocyst stage they are unable to re-enter normal development due to specific loss of the epiblast (Nichols et al., 2001). This demonstrates a requirement for signalling through gp130 to maintain the pluripotent compartment during diapause that may relate to its function in capturing and maintaining the pluripotent state in the form of ES cells in culture. Thus, the responsiveness of ES cells to LIF may have its origins in the phenomenon of diapause. This raises the possibility that LIF signalling will influence the epiblast and the establishment of ES cells only in species which exhibit diapause and may explain why hES cells are unresponsive to LIF (Thomson et al., 1998; Daheron et al., 2004; Humphrey et al., 2004). However, the true test of human embryo responsiveness to LIF cannot be performed on established hES cell lines because these cells appear to correspond to a distinct developmental stage (Tesar et al., 2007).

LIF is not strictly sufficient for self-renewal since the addition of serum to the medium is still required to support self-renewal. Upon removal of serum from the culture medium mES cells undergo neural differentiation, even in the presence of LIF. This prompted Ying *et al* to propose that inhibition of neural differentiation might support serum-free self-renewal. Bone Morphogenetic Proteins (BMPs) were

known to inhibit neural differentiation and it was demonstrated that addition of BMP or the related GDF to serum-free medium containing LIF could support self-renewal (Ying et al., 2003a). The BMP pathway signals through SMAD1, 5 and 8 to induce expression of ID proteins. Constitutive expression of ID1, 2 or 3 is sufficient to replace the requirement for BMP or serum.

mES cells can now be derived and cultured in defined conditions (although the serum- derived albumin normally used in these conditions is not fully defined) and cells cultured under these conditions retain the ability to contribute to the developing embryo and to all tissues of the resulting adult mouse, including the germ line (Ying et al., 2003a). However, these experiments were restricted to 129 and 129-C57B6 F1 mice and have not been demonstrated to support derivation from the recalcitrant C57B6 and CBA mouse strains.

1.3.2 ERK1/2 Signalling

Mitogen activated protein kinases (MAPKs) connect cell surface receptor molecules with their down-stream effectors by means of a cascade of phosphorylation (reviewed in (Katz et al., 2007)). They have been implicated in the control of virtually all cellular processes. Extracellular signal-regulated protein kinase-1 and -2 (ERK1/2) are two MAPKs implicated in the regulation of ES cell self-renewal and in early embryonic cell fate choice. ERK1/2 function downstream of MEK1/2 and are activated in response to many extracellular cues including FGFs. FGF ligands engage with FGF receptors resulting in activation of the receptor tyrosine kinase activity. SH2 domain containing proteins including Shp2 and Grb2 are recruited and in turn recruit SOS which activates Ras and initiates the MAPK cascade to activate ERK1/2. Activated ERK1/2 can act on cytoplasmic targets and can also translocate to the nucleus where it activates various TFs (Marais et al., 1993).

A negative role for ERK1/2 in self-renewal was indicated in a study demonstrating that mutation of the Shp2 binding site of the gp130 receptor, which prevents activation of the ERK1/2 pathway by LIF, significantly reduces the concentration of

cytokine required to support self-renewal (Burdon et al., 1999b). In fact, Shp2 is dispensable for ES cell self-renewal as ES cells lacking functional Shp2 were established. These cells demonstrated compromised differentiation and an increased sensitivity to LIF (Qu and Feng, 1998). Similarly, ES cells lacking functional Grb2, which couples to SOS to activate Ras, can be derived and show a defect in endodermal differentiation (Cheng et al., 1998). It has also been shown that a MEK-inhibitor PD98059 (which inhibits activation of ERK1/2) inhibits differentiation as judged by the persistence of *Oct4* expression during EB differentiation (Burdon et al., 1999b). This study suggested that ERK signalling promotes differentiation and that LIF-mediated activation of gp130 therefore results in the activation of pathways with opposing effects on self-renewal. Altering the balance between STAT3 and ERK1/2 signalling in favour of STAT3 promoted self-renewal. More recently a novel small molecule, named Pluripotin, was identified and demonstrated to be capable of supporting LIF-independent self-renewal (Chen et al., 2006). It is believed to act in part by the inhibition of ERK1/2 phosphorylation. Reduced ERK1/2 signalling is also likely to underlie the enhanced self-renewal seen in ES cells lacking *Fgf4* (Wilder et al., 1997), since the high levels of FGF4 secreted by ES cells result in auto-stimulation of the ERK1/2 cascade (Kunath et al., 2007). Furthermore, ablation of suppressor of cytokine signalling 3 (SOCS3), which is a downstream target of STAT3 mediating negative feedback regulation of gp130, results in an increase in differentiation. This is a surprising result since SOCS3 mediates negative feedback and inhibits STAT3 signalling but the authors attribute the phenotype to an alteration in the balance of STAT3 and ERK1/2 signalling in favour of the ERK1/2 cascade (Forrai et al., 2005), presumably because SOCS3 normally has a strong inhibitory effect on the MAPK pathway.

More recent work proposes that the ERK signal promotes the transition of ES cells into a state competent to respond to differentiation cues (Kunath et al., 2007; Stavridis et al., 2007). ES cells lacking *Fgf4* or *Erk2* failed to differentiate under conditions that normally promote neural differentiation or in response to BMP4 which promotes non-neural differentiation (Kunath et al., 2007). These cells activate the early differentiation marker *Fgf5* in the absence of LIF but this was fully

reversible and does not therefore represent a true differentiation event. *Fgf4* null ES cell differentiation could be rescued by the addition of recombinant FGF4 demonstrating that the defect could be attributed to a loss of autocrine FGF4 signalling (Kunath et al., 2007). It was also shown that a discrete period of FGF signalling was sufficient to allow differentiation supporting the idea that ERK signalling acts as a trigger for differentiation (Stavridis et al., 2007).

Observations in ES cells are consistent with findings in the preimplantation embryo. A detailed examination of the localization of active ERK in the early post-implantation embryo shows that pERK is confined to extraembryonic tissues with the exception of dividing cells of the epiblast (Corson et al., 2003). *Grb2-/-* embryos fail to form primitive endoderm (PrE) and the mutually exclusive pattern of Gata6 and Nanog, which mark PrE and epiblast progenitors respectively, in the E3.5-4.5 embryo is lost with Nanog expression becoming uniform in all the cells of the ICM (Chazaud et al., 2006). The phenotype of *Fgf4-/-* embryos and ES cells (Wilder et al., 1997) is consistent with this although a detailed examination of the lineage markers has not been carried out in vivo. Furthermore, pharmacological inhibition of ERK signalling in 8 cell embryos prevents the formation of PrE in favour of an expanded, homogeneously Nanog positive ICM (Nichols and Silva, unpublished). The emerging picture is that autocrine FGF4 signalling activates ERK1/2 through Grb2 and MEK leading to the formation of PrE in the embryo and promoting differentiation in ES cells. The downstream effectors have not yet been identified but it has been reported that ES cells prevented from undergoing endodermal differentiation by FGFR inhibition can be induced to differentiate by the forced expression of Gata factors (Fujikura et al., 2002) suggesting that Gata-4 and/or -6 might be downstream targets of the ERK signal. Alternatively, the ERK cascade may act to down-regulate *Nanog*, permitting expression of Gata factors, as has been suggested from studies in ES cells (Hamazaki et al., 2006).

1.3.3 PI3K Signalling

Phosphoinositide 3-kinase (PI3K) phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5) triphosphate (PIP3) which acts as a second messenger, binding proteins with pleckstrin homology (PH) domains including protein kinase B (PKB, also known as AKT) and 3-phosphoinositide-dependent protein kinase (PDK1). PDK1 phosphorylates and activates PKB, p70 ribosomal S6 kinase (S6K) and p90 ribosomal S6 kinase (RSK) (Williams et al., 2000) which mediate a number of downstream effects. PKB is the best studied of these targets in ES cells. Activation of PKB requires phosphorylation by PDK1 on Thr308 as well as PI3K-dependent phosphorylation at Ser473 (Alessi et al., 1996). PKB regulates many downstream targets including glycogen synthase kinase 3 (GSK3) (Alessi and Cohen, 1998) and mammalian target of rapamycin (mTOR) (reviewed in (Hay and Sonenberg, 2004)) through inhibition of the tuberous sclerosis complex (TSC) (Manning and Cantley, 2003). PI3K is activated by many growth factors and cytokines including LIF and insulin which is an important component of serum-free growth conditions and its activity has been shown to be important for cell survival and proliferation in many different cell types (Paling et al., 2004; Takahashi et al., 2005).

The PI3K pathway has also been implicated in growth and survival of ES cells. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) negatively regulates PI3K signalling by dephosphorylating PIP3. ES cells lacking *Pten* exhibit increased proliferation rate and reduced serum-dependence (Sun et al., 1999). Creating a double knockout of *Pten* and the ubiquitously expressed *Pkb* isoform, *Akt1*, resulted in ES cells with PKB activity close to that of wild-type controls. *Pten/ Akt1* double knockout ES cells had a much slower proliferation rate than *Pten* null ES cells, slower even than wild-type cells, indicating that PKB was the key downstream effector in the *Pten* knockout (Stiles et al., 2002). Surprisingly, ES cells lacking PDK1, which is required for activation of PKB, did not exhibit reduced proliferation (Williams et al., 2000) suggesting that PKB is dispensible in ES cells, at least in the context of serum-containing culture media. It is possible that a phenotype would become apparent if these cells were exposed to culture conditions lacking serum or serum-replacement, both of which may provide alternative growth and

survival signals. A novel, ES-specific form of RAS (Eras) has been demonstrated to confer constitutive activity on the PI3K signalling pathway (Takahashi et al., 2003). Deletion of *Eras* results in decreased proliferation and tumorigenicity of ES cells consistent with the *Pten* and *Pkb* knockout phenotypes (Stiles et al., 2002). However, mice lacking *Eras* are viable and fertile demonstrating that it is not essential (Takahashi et al., 2003). An *in vivo* role for PTEN was demonstrated by conditionally deleting *Pten* in the PGCs. This lead to testicular teratomas in all male mice and facilitated the derivation of pluripotent EG cells (Kimura et al., 2003). These observations are consistent with increased cell survival and proliferation in *Pten* null cells in the germ-line. It is not clear if the enhanced PI3K activity predicted in these cells (Sun et al., 1999) facilitates the acquisition of pluripotency during the derivation of EG cells from PGCs but recent work suggests that PKB is the key downstream effector and that its effect may be mediated through the inhibition of p53 (Kimura et al., 2008). This effect could simply be an enhancement of cell survival but it has been suggested that p53 can induce differentiation by down-regulation of Nanog (Lin et al., 2005). Inhibition of p53 and upregulation of Nanog would be predicted to enhance the establishment of pluripotent cells but this remains to be investigated. Activated AKT is also able to mediate self-renewal of GS cells but the primary effect appears to be the enhancement of proliferation (Lee et al., 2007). Interestingly, the spontaneous formation of mGS cells (Kanatsu-Shinohara et al., 2004) was not observed in cultures with activated AKT signalling suggesting that the low frequency conversion of GS cells to multipotency is not driven by this signalling pathway (Lee et al., 2007).

mTOR regulates protein synthesis at least in part through the phosphorylation of p70 S6 Kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP1). PI3K signalling can positively regulate mTOR and it is therefore an attractive candidate for a downstream effector. Embryos lacking *mTor* appear normal at the blastocyst stage but cannot give rise to ES cells when plated in culture. Conditional deletion of *mTor* in ES cells results in severe growth retardation. Again, this phenotype is consistent with the phenotypes of mutations affecting the PI3K pathway. The studies summarised here support a role for PI3K signalling in the

proliferation of ES cells but do not imply an instructive role in directing self-renewal. On the contrary, the fact that ES cells lacking the key signalling components PKB and PDK1 can be established and maintained implies that signalling through this pathway is non-essential for self-renewal. However, several studies suggest that PI3K may act to suppress differentiation.

Inhibition of PI3K using either the inhibitor LY294002 or a dominant negative subunit of PI3K resulted in differentiation even in the presence of LIF (Paling et al., 2004). The authors attributed this to an upregulation of ERK activity as the differentiation could be rescued by the simultaneous inhibition of MEK. It has also been shown that expression of a constitutively active form of PKB can confer LIF-independence on ES cells (Watanabe et al., 2006). However, this study relies heavily on short-term assays and offers no mechanism for the suppression of differentiation. This work is supported to a degree by the identification of a small molecule, Pluripotin, that supports self-renewal through up regulation of PI3K signalling together with inhibition of ERK1/2 signalling (Chen et al., 2006) but it remains unclear what the critical contribution of PI3K upregulation is and whether this compound has any off-target activity. A possible mechanism for the action of PI3K is the inhibition of GSK3 through phosphorylation by PKB since GSK3 inhibitors have been implicated in the maintenance of pluripotency (Sato et al., 2004). In support of this Storm *et al* showed that differentiation induced by PI3K inhibition can be rescued by simultaneous inhibition of GSK3 (Storm et al., 2007). They show that while Nanog levels decrease in response to PI3K inhibition, GSK3 inhibition maintains levels of Nanog. However, there is no evidence that PI3K inhibition results in increased GSK3 activity in ES cells or that Nanog is directly regulated by factors downstream of GSK3 or PI3K. Furthermore, ES cells have been generated that express a form of GSK3 that is insensitive to phosphorylation by PKB (McManus et al., 2005) demonstrating that inhibition of GSK3 by PKB is not critical in ES cell self-renewal.

1.3.4 Canonical Wnt Signalling

Binding of Wnt ligands to the cell surface receptor Frizzled results in signalling through Dishevelled to inactivate the destruction complex which is responsible for phosphorylating β-catenin and targeting it for proteolysis. Inhibition of the destruction complex results in stabilization and nuclear accumulation of cytoplasmic β-catenin. The destruction complex kinase responsible for β-catenin phosphorylation is GSK3 so direct inhibition of GSK3 can mimic Wnt signalling (reviewed in (Patel et al., 2004)). However, GSK3 phosphorylates many targets other than β-catenin so its inhibition is predicted to have pleiotropic effects (reviewed in (Doble and Woodgett, 2003)).

Canonical Wnt signalling has been implicated in the regulation of ES cell differentiation but reports are often conflicting (Haegele et al., 2003; Otero et al., 2004), perhaps as a result of slightly different experimental contexts. The identification of a novel GSK3 inhibitor, BIO, and the claim that it can support self-renewal (Sato et al., 2004) has given rise to the idea that Wnt signalling can support the self-renewal of pluripotent cells. The demonstration that BIO has a similar effect on both mouse and human ES cells also suggested that this was the first signalling pathway capable of supporting self-renewal in common between the two species. However, the initial work using this compound did not demonstrate long-term propagation of ES cells and as with all pharmacological inhibitors the specificity was questioned. Several reports have however claimed that Wnt3a can support self-renewal (Hao et al., 2006; Ogawa et al., 2006; Sato and Brivanlou, 2006) and show that this effect is mediated by β-catenin through the use of constitutively active forms of β-catenin (Ogawa et al., 2006). However, these studies rely heavily on the use of Wnt3a-conditioned media (CM) and on undefined culture conditions employing serum and/or feeders. The finding that CM but not recombinant Wnt3a could support self-renewal suggested that the CM contained additional factors (Ogawa et al., 2006). The CM was found to weakly activate a STAT3-reponsive luciferase reporter suggesting that it contained activity similar to low concentrations of LIF. Subsequently it was demonstrated that low LIF concentrations, normally not capable of supporting self-renewal, synergised with recombinant Wnt3a to support long-term self-renewal. The same group found that BIO also activated a STAT3-responsive

reporter suggesting that it had non-specific effects likely to act together with β-catenin to promote self-renewal.

Interestingly ES cells totally lacking in GSK3 (Doble et al., 2007), and therefore with high levels of constitutively active β-catenin, show a degree of resistance to differentiation. Under normal self-renewal conditions the cells have high background differentiation, probably as a result of expression of lineage-associated genes, but when induced to differentiate ES cells persist for much longer than wild-type controls. This phenotype suggests that targets downstream of GSK3 can promote differentiation while others resist differentiation, perhaps by stabilization of the ES cell state (Arias and Hayward, 2006).

1.4 Human Embryonic Stem Cells

hES cells were first derived in 1998 (Thomson et al., 1998). They were derived in conditions almost identical to those originally used to derive mouse ES cells but it was immediately clear that they could not be cultured under the same feeder-free conditions that support mES cells - the addition of recombinant LIF to the culture medium was not sufficient to inhibit differentiation (Thomson et al., 1998; Reubinoff et al., 2000; Daheron et al., 2004; Humphrey et al., 2004). Since 1998 many groups have developed conditions for the growth of hES cells, often attempting to develop systems free of animal-derived products (Amit et al., 2003; Amit et al., 2004). Perhaps the only universally accepted method for culturing hES cells is on a feeder layer of mouse embryonic fibroblasts, in serum- or serum replacement-containing medium supplemented with basic fibroblast growth factor (FGF2). While there have been some demonstrations of hES cell culture in defined conditions these seem to rely on non-physiological concentrations of FGF2 or have not been demonstrated to support derivation of hES cells *de novo*. The molecular mechanisms that mediate the self-renewal signals derived from these culture conditions remain unknown.

When investigating hES cells it is important to bear in mind some of the limitations as compared to their mouse counterparts. Firstly, hES cells survive poorly when

dissociated into single cells and exhibit poor cloning efficiency. This makes rigorous tests of their ability to self-renew in the absence of autocrine signals difficult to test. Secondly, hES cells cannot be tested for their ability to contribute to chimeras as their mouse counterparts can. This is considered the most rigorous test of 'normality' for mES cells and in its absence hES cell culture conditions should be tested for their ability to support derivation *de novo* if they are to be considered robust conditions for hES cell culture. Furthermore, a normal karyotype and the ability to differentiate into cell types representative of all three germ layers should be maintained through an extended period of *in vitro* culture

1.4.1 Intracellular Determinants

Although hES cells differ from mES cells in the expression of certain cell surface markers they express the core TFs of pluripotency; OCT4, SOX2 and NANOG. The expression of these core factors suggests conservation of the mechanisms governing pluripotency despite the differences in culture requirements. However, global studies identifying targets of the core TFs in m and hES cells did not show a significant overlap between the two species. One explanation for these differences was recently revealed by the identification of a pluripotent cell type originating from the mouse post-implantation epiblast, a slightly later developmental stage to the blastocyst from which ES cells are derived. Through global transcriptional profiling hES cells were found to be more similar to mouse EpiSCs than ES cells. Although hES cells are derived from blastocyst-stage embryos it seems likely that the established cell lines correspond to a later developmental stage. If this is the case it provides a rationale for the reliance of hES cells on FGF2 and the lack of response to LIF.

Analysis of the intracellular determinants of pluripotency in hES cells has been limited to date with work focusing on defining the extracellular signals. As genetic experiments cannot be carried out on the early human embryo, confirming the role of the key transcription factors in early embryonic development is impossible. Studies will be limited to the effect of their manipulation *in vitro* on the behaviour of hES

cells. Studies to date seem to imply similar roles for Oct4 and Nanog in self-renewal of hES cells to those characterised in mES cells.

Nanog expression in the embryo has been examined by immunostaining and is found to be absent from the unfertilized egg and the early embryo with expression in the blastocyst tightly restricted to cells of the ICM. RNAi knock-down of Nanog expression in hES cells resulted in increased differentiation and upregulation of markers of trophectoderm and extraembryonic endoderm (Hyslop et al., 2005; Zaehres et al., 2005). This finding is consistent with the proposed role of Nanog in mES cells in inhibition of differentiation into primitive endoderm (Mitsui et al., 2003) although trophectoderm differentiation is normally observed in mES cells upon down-regulation of *Oct4* (Niwa et al., 2000). The effect of Oct4 knock-down in hES cells has been examined and results in clear differentiation with some evidence of upregulation of TE markers (Matin et al., 2004; Zaehres et al., 2005). Furthermore, overexpression of Nanog in hES cells supported culture in the absence of feeders or conditioned medium (Darr et al., 2006). However, this group also report that forced Nanog expression promotes a primitive-ectoderm-like phenotype in these cells. This is a surprising finding given that no such phenotype is observed in the mouse and that, with the benefit of hindsight, we know hES cells to correspond more closely to cells of the primitive ectoderm than cells of the ICM (Tesar et al., 2007).

1.4.2 Extracellular Signals

Most hES cells are routinely cultured on feeders or in feeder-conditioned medium (Thomson et al., 1998; Xu et al., 2001; Amit et al., 2003). In feeder-free systems substrates such as Matrigel are used for cell attachment (Xu et al., 2001). Where serum is not used commercially available serum-replacement usually is. All of these components are sources of undefined signals. The best established medium supplement capable of supporting hES cell growth is FGF2 but how the signalling pathways downstream of FGF2 contribute to self-renewal remains unknown. Recently James Thomson's group provided the most convincing demonstration to date of hES cell self-renewal in defined conditions, including the derivation of two

new lines (Ludwig et al., 2006). However, their culture conditions were complex, with five different growth factors added, including high concentrations of FGF2, and a matrix composed of four different components used to aid cell attachment. Other signals have been implicated in the maintenance of hES cells, such as Wnt (Sato et al., 2004) and Activin (Vallier et al., 2004; James et al., 2005), but their requirement for self-renewal or their ability to support self-renewal requires more in depth investigation. While advances have been made in our ability to derive and culture hES cells under defined, xeno-free conditions our understanding of the signalling pathways that support self-renewal remains poor.

1.4.2.1 LIF/JAK/STAT

Addition of exogenous LIF is sufficient to maintain mES cells in the presence of serum (Smith et al., 1988; Williams et al., 1988) or BMP (Ying et al., 2003a). The addition of LIF to hES cells is not however sufficient to support self-renewal. Several groups have reported that hES cells possess the machinery to respond to LIF and that addition of exogenous LIF leads to activation of STAT3 (Daheron et al., 2004; Humphrey et al., 2004), the essential downstream component of the LIF pathway in mES cell self-renewal (Niwa et al., 1998; Matsuda et al., 1999). However, the level of activation of STAT3 in hES cells is lower and the published results fail to establish that STAT3 is functional in hES cells. Furthermore, the effect of inhibiting STAT3 signalling has yet to be investigated in hES cells. While it remains to be demonstrated if hES cells can self-renew in the absence of any STAT3 activity, and it remains to be rigorously tested if functional STAT3 signalling can affect self-renewal, the question of whether this pathway has any significant role to play in hES cell self-renewal remains open. The recent finding that hES cells correspond more closely to mouse EpiSCs than ES cells and that EpiSCs are also unresponsive to LIF (Brons et al., 2007; Tesar et al., 2007) supports the idea that hES cells are a fundamentally different cell type to mES cells and will not respond to LIF.

1.4.2.2 FGF2

In the initial derivation of hES cells FGF2 was not added to the culture medium but it was identified soon thereafter as supportive of hES cell culture. FGF2 is now routinely added to the media for culture of hES cells though its role in promoting self-renewal remains undefined. It has been reported that hES cells produce several isoforms of FGF2 capable of activating both intracrine and autocrine pathways (Dvorak et al., 2005). The authors demonstrate that the low molecular weight isoform is secreted into the medium and that inhibition of signalling through FGFRs results in differentiation of hES cell colonies, characterized by the appearance of flat, alkaline phosphatase-negative cells in the centre of the colonies. Inhibition of the FGFRs is accompanied by a reduction in phosphorylation of mitogen activated protein kinase kinase (MEK1/2) and their substrates ERK1/2, but the authors do not claim that this is the direct cause of the differentiation. Addition of recombinant FGF2 did not affect the proliferation of the cells but at higher concentrations (10/20ng/ml) resulted in a change in morphology to more tightly packed colonies lacking the flattened cells often observed around the edge of the colonies. This work provides evidence for the requirement for FGF-signalling to maintain hES cells but the molecular pathways involved remain undefined.

More recently several groups have advanced towards completely defined culture conditions for hES cell culture by increasing the concentration of FGF2 as high as 100ng/ml compared to a supplement of 4ng/ml used when cells are cultured on feeders. It has been suggested that the reason for this could be trivial – that the stability of FGF2 is lower in unconditioned medium and that higher concentrations must therefore be added to maintain a minimum threshold of FGF2 and prevent exposure of the cells to periods of FGF2 deprivation. Another group has suggested that the combination of high FGF2 and BMP inhibition by Noggin can support feeder-free self-renewal. This finding may be specific to the conditions employed by this group which showed a high level of basal BMP signalling – a balance of signals must be maintained to support self-renewal as demonstrated for mES cells where BMP signals act to support self-renewal only in the context of media supplemented with LIF.

FGFs can activate multiple signalling pathways including PI3K-AKT, MAPK-ERK, and PLC-PKC. All of these are likely to affect the growth of ES cells and the balance between self-renewal and differentiation that exists in any ES cell culture. Dissecting these pathways and identifying the critical components for self-renewal should further our understanding of the basic biology of these cells. FGF2 has been used in the derivation of mouse EG cells, mGS cells (although it is not required once the lines are established) and in the derivation and culture of mouse EpiSCs suggesting a more general role for FGF signalling in promoting stem cell growth *in vitro*. FGFs are known to promote cell growth and proliferation.

1.4.2.3 Canonical Wnt Signalling

As discussed above, Wnt ligands and GSK3 inhibitors are capable of mediating the stabilisation of β-catenin and its subsequent nuclear accumulation allowing it to interact with cofactors and activate transcription of target genes. It was demonstrated by Sato *et al* that a pharmacological inhibitor of GSK-3β could support self-renewal of both mES and hES cells (Sato et al., 2004). However, their assay examined growth over a short period and did not demonstrate self-renewal following passaging of the cells. Another study suggests that Wnt/β-catenin signalling promotes survival/ proliferation of hES cells but does not promote self-renewal over longer periods (Dravid et al., 2005). The data suggests that Wnt-signalling during a 4 day culture period in feeder-free conditions actually reduces the proportion of cells capable of forming alkaline-phosphatase positive colonies when the cells are seeded back onto feeders. Furthermore, 26 days of culture in the presence of Wnt-antagonists did not abolish the formation of AP+ colonies demonstrating that Wnt-signalling is not required for self-renewal. Canonical Wnt signalling is likely to affect the balance of self-renewal and differentiation in hES cells and effects on cell metabolism are anticipated, particularly when GSK3 inhibitors are employed. However, there is little evidence for a long-term self-renewal effect of this pathway on hES cells.

1.4.2.4 TGFβ/Activin/Nodal

TGFβ/Activin/Nodal signal through the TGFβ super-family of receptors to activate downstream signalling including phosphorylation of Smad2 and Smad3 (reviewed in (Shi and Massague, 2003)). Several groups have reported a positive role for TGFβ-mediated signalling in the maintenance of pluripotency in hES cells (Amit et al., 2004; Vallier et al., 2004; James et al., 2005). While Amit *et al* present no mechanism they demonstrate long-term self-renewal in a feeder- and serum-free system. Cells were cultured on a fibronectin substrate in media containing serum-replacement, TGFβ, LIF and FGF2 (Amit et al., 2004). Their results suggest that these conditions support self-renewal with efficiency approaching that seen when hES cells are grown on feeders. The interpretation of their findings is however complicated by the presence of the undefined serum-replacement. Another recent paper reports the chance finding that Activin A can maintain self-renewal in combination with keratinocyte growth factor (KGF) and nicotinamide, which promote growth and survival of the cells(Beattie et al., 2005). Addition of the activin inhibitor follistatin to cultures on MEFs induced differentiation. This, in combination with the demonstration that MEFs secrete activin A into the culture medium, suggests that TGFβ-signalling may underlie (at least in part) the ability of MEFs to support undifferentiated growth of hES cells. This finding is supported by a report that pharmacological inhibition of Smad2/3 phosphorylation results in differentiation of hES cells cultured on Matrigel in MEF-CM (James et al., 2005). Furthermore, addition of Activin A to non-conditioned, chemically defined medium lacking serum-replacement was found to maintain expression of pluripotency markers although in this case addition of Activin A was not sufficient for long-term maintenance of undifferentiated cultures. Long-term propagation of hES cells was achieved by supplementing defined media with both FGF2 and Activin or Nodal, coating tissue culture dishes in serum to aid cell attachment (Vallier et al., 2004).

The work described above is based on established hES cell lines and lacks the demonstration that these growth conditions can support *de novo* derivation of ES cells, the most stringent test that can be applied in the human system. However, two

groups recently returned to the mouse with conditions optimised for hES cell culture. The subsequent derivation of EpiSCs from both the mouse and the rat suggests that these growth conditions do support the propagation of a distinct type of pluripotent cell (Brons et al., 2007; Tesar et al., 2007).

1.5 Scope of the Project

ES cells are an *in vitro* phenomenon; the cells to which they are most closely related, the cells of the pluripotent epiblast, exist only transiently *in vivo*. Mouse (m) ES cells have only been established under defined conditions for the inbred 129 strain (Ying et al., 2003a) while high efficiency derivation from C57Bl6 and CBA strains has only been achieved through the technically demanding procedure of microdissection and culture in the presence of serum and feeders (Brook and Gardner, 1997). Furthermore, while human (h) ES cells have been derived (Thomson et al., 1998), it is clear that these cells do not respond to the culture conditions established for mES cells (Thomson et al., 1998; Daheron et al., 2004; Humphrey et al., 2004) and may in fact represent a distinct cell type (Tesar et al., 2007). This, together with the repeated failure to establish ES cells from rats or other mammalian species suggests that mES cells are the exception and that it may not be possible to establish ES cells that behave similarly to those described for the mouse from other species. Mice may simply be unique or it may be that researchers are limited by the established culture conditions, developed in the artificial environment of *in vitro* tissue culture. It is hoped that by better understanding the nature of mES cells and the signalling pathways involved in their self-renewal our understanding might be extended to other species, in particular human.

To this end it has been the long-term research interest of our group to develop fully defined culture conditions for mES cells that facilitate investigation of the molecular mechanisms governing self-renewal. A recently developed culture media based on a combination of three pharmacological inhibitors (named ‘3i’) has been demonstrated to support robust self-renewal. The aim of this project is to define the requirements for the individual inhibitors used and to elucidate the underlying mechanisms

governing self-renewal. I demonstrate that 3i relieves mES cells from their dependence on signals previously thought essential for their propagation. It is our hope these culture conditions will support the derivation and propagation of ES cells from other mammalian species.

Chapter 2. Materials and Methods

2.1 Cell Culture Reagents

2.1.1 Media and Supplements

GMEM Complete:	Glasgow Minimal Essential Medium (GMEM) Foetal Calf Serum (10%) Non-essential amino acids (Gibco, 11140-035) L-Glutamine, 2mM (Gibco, 25030-024) Sodium Pyruvate, 1mM (Gibco, 11360-039) 2-mercaptoethanol LIF (100U/ml)
N2B27:	1:1 Neurobasal:DMEM:F-12 (Gibco, 21331-020) L-Glutamine, 2mM (Gibco, 25030-024) N2, 1:200 B27, 1:100 (Gibco, 17504-044) 2-mercaptoethanol
B27-free medium:	As N2B27 but without B27 and using 1:100 N2
Insulin-free medium:	As B27-free but using N2 supplement made without insulin
3i:	N2B27 supplemented with: PD184352, 0.8μM SU5402, 2μM CHIR99021, 3μM
2i:	N2B27 supplemented with: PD0325901, 1μM

CHIR99021, 3μM

PS: N2B27 supplemented with:
PD184352, 0.8μM
SU5402, 2μM

N2 (100x): Insulin (Sigma I-1882) 2.5mg/ml
Apo-transferrin (Sigma T-1147) 10mg/ml
BSA (Gibco 15260-037) 7.5mg/ml
Progesterone (Sigma P8783) 0.002 mg/ml
Putrescine (Sigma P5780) 1.6mg/ml
Na Selenite (Sigma S5261) 3μM
Dissolve in DMEM:F12 (Gibco 21331-020)

B27 (50x): Purchased from Gibco (17504-044). See Brewer *et al*; J. Neurosc. Res. 35(5): 567-76 (1993)
Biotin
L-carnitine
Ethanolamine
D+ Galactose
Putrescine
Selenium
Catalase
SOD
Linoleic acid
Linolenic acid
Progesterone
Retinylacetate
Tocopherol
Tocopherolacetate
BSA
Transferrin

Insulin
Corticostereon
Tri-iodothyronine

rHSA: Recombinant human serum albumin (Cellastim, Invitria) used in place of BSA in N2. Final concentration in media: 37.5µg/ml

2.1.2 Cytokines Growth Factors and inhibitors

LIF: 100U/ml. Conditioned media generated by transient transfection of Cos7 cells with plasmid encoding human LIF and harvesting conditioned media after 4 days.

BMP4: Recombinant human, 10ng/ml (R&D systems, 314-BP-010)

EGF: Recombinant human, 10ng/ml (Peprotech, 100-15)

FGF2: Recombinant human, 10ng/ml (Peprotech, 100-18B)

Wnt3a: Recombinant mouse, 100ng/ml (R and D systems, 1324-WN-002)

Inhibitors: PD184352 (Signalling Technologies, University of Dundee)
SU5402 (Calbiochem, 572630)
CHIR99021 (Signalling Technologies, University of Dundee)
PD173074 (Calbiochem, 341607)
PD0325901 (Signalling Technologies, University of Dundee)
SB216762 (Sigma, S3442)
SB415286 (Sigma, S3567)

2.1.3 Other Tissue Culture Reagents

Antibiotics: Puromycin (Sigma, P9620): 1.0µg/ml, 0.5µg/ml serum-free, 2.0µg/ml for episomal transfections
Hygromycin B (Gibco, 10687-010): 150µg/ml, 100µg/ml serum-free
Zeocin (Gibco, R250-01): 200µg/ml

Gelatine (0.1% in PBS)

Trypsin: 0.25% Trypsin (Gibco, 15090-046)
1.3mM EDTA
0.1% Chick Serum (Flowlabs)
In PBS

PBS (Sigma)

2.1.4 Cell lines

E14Tg2a
ES cell line derived from 129/Ola mice.

E14 1V C
Subclone of E14Tg2a, tested for germ-line completency.

E14T
E14Tg2a derived cells which maintain a stable integration of the large T-antigen under G418 selection.

NanogT

E14Tg2a derived cells which maintain a stable integration of the large T-antigen under G418 selection and *Nanog* under puromycin selection.

Oct4GIP

Derived from parental line CGR8. Stably express GFP and puromycin resistance under control of the *Oct4* promoter (Ying et al., 2002). Addition of puromycin selects for *Oct4*-expressing ES cells.

BT12

E14Tg2a-derived ES cells carrying a targeted deletion of *Nanog* (Chambers et al., 2007). Express hygromycin resistance under the control of the endogenous *Nanog*-promoter. ES cells can be selected for using hygromycin.

44cre6

Independently E14Tg2a-derived *Nanog*-null cell line (Chambers et al., 2007).

***Fgf4*-heterozygous and –null**

Obtained from Rizzino lab (Wilder et al., 1997). ES cells carrying targeted knockout of one or two *Fgf4* alleles.

GSK3 double knockout

Obtained from Bradley Doble and James Woodgett (Doble et al., 2007). ES cells with targeted knockout of both alleles of *Gsk3-α* and *–β*.

***β-catenin*-heterozygous and -null**

Obtained from Kemler group. ES cells carrying targeted knockout of one or both *β-catenin* alleles. Heterozygous line has one null and one floxed allele. Null cells can be generate by expression of Cre recombinase.

***Ecad*-null**

Obtained from Kemler group (Larue et al., 1996). ES cells carrying targeted knockout of both *Ecad* alleles.

Bcl2IP

E14Tg2a ES cells stably expressing Bcl2 under control of CAG promoter. Puromycin resistant.

MTIP

E14Tg2a ES cells carrying a stable integration of pPy Floxed MT IP gfp (AGS 1126). Puromycin resistant.

Y118F

E14Tg2a ES cells carrying a stable integration of pCAGgrgpY118FIZ (AGS 796). Can be maintained in media supplemented with GCSF instead of LIF. Zeocin resistant.

Klf4IH

E14Tg2a ES cells carrying a stable integration of pPyCAG KLF4 IH (AGS 1233). Hygromycin resistant.

2.2 Cell Culture

2.2.1 Tissue Culture Routine

All cell lines were maintained in 37C, humidified incubators maintained at 7% CO2. For low oxygen experiments incubators were connected to a source of nitrogen and set to 5% O2.

2.2.1.1 Mouse Embryonic Stem Cells

mES cell lines were routinely cultured on gelatine-coated tissue culture flasks/plates (Iwaki) in GMEM complete media. Routine serum-free culture was performed using

N2B27 supplemented with LIF and BMP4. Media was changed daily and cells were passaged when approaching confluency. To passage, cells were washed in phosphate buffered saline (PBS), incubated with trypsin for approximately 2 minutes or until cells detached, resuspended in 5-10 trypsin volumes of media, harvested by centrifugation (1300rpm, 3mins) and replated at the required density.

2.2.1.2 Mouse Neural Stem Cells

Mouse NS cells were derived by culturing ES cells as aggregates for 10-14 days in N2B27 alone before plating onto gelatine-coated plates in the presence of EGF and FGF. NS-like cells outgrew from attached aggregates and were thereafter maintained in NS cell conditions as described (Conti et al., 2005). Briefly, NS cells were maintained on gelatine-coated plates in NS-A medium (Euroclone) supplemented with EGF and FGF2, both 10ng/ml. Cells were passaged every 2-3 days by trypsinisation.

2.2.1.3 Other cell lines

Other cell lines, including Cos7 and MEFs, were cultured as for ES cells but LIF was not included in the media.

2.2.2 Substrates

2.2.2.1 Gelatine

Flasks/plates were covered with 0.1% gelatine in PBS for a minimum of 20 minutes at room temperature. Gelatine was aspirated and cells plated immediately.

2.2.2.2 Laminin

Flasks/plates were covered with poly-L-ornithine and incubated at room temperature for 1hr. Poly-L-ornithine was aspirated and flasks/plates washed twice in PBS. Flasks/plates were then covered with laminin and incubated at 37C for at least 1hr (overnight is fine). Laminin was aspirated and replaced with PBS/media until ready to use.

2.2.3 Freezing

For long-term storage mESCs can be frozen and stored in liquid nitrogen. Cells were harvested by trypsinisation and 2-3 million cells resuspended in 0.5ml media containing 10% dimethyl-sulphoxide (DMSO) and placed in cryovials (Nunc, 377224). Vials were placed immediately at -80C and after overnight storage transferred to liquid nitrogen.

2.2.4 Feeders – Mouse Embryonic Fibroblasts

Where stated, mESCs were grown on a mitotically inactivated layer of mouse embryonic fibroblasts (MEFs). MEFs were prepared from mid-gestation embryos. Embryos were dissociated mechanically and by trypsinisation before plating on gelatine-coated plastic in GMEM complete (no LIF). The outgrowing cells were passage 1 MEFs. For use as feeders confluent MEFs were mitotically inactivated by exposure to 10μg/ml Mitomycin C (Sigma, M4287) for 3 hours and then trypsinised and replated in the desired plates and flasks at a density equivalent to the confluent flask (approximately $1x10^5$ cells/cm^2).

2.3 Manipulation of Cells

2.3.1 Stable Transfections – Electroporation

In order to stably integrate transgenes into mESCs plasmids encoding the desired construct were linearized by restriction digest (enzyme sca1 or other appropriate single-cutting enzyme), purified by ethanol precipitation and electroporated into cells. 10 million cells per transfection were harvested by trypsinisation, washed in PBS, resuspended in 800µL PBS and placed in an electroporation cuvette (Biorad, 165-2081EDU). 50µg linearized DNA was added to the cuvette. Cells were then electroporated (Cap 3.0µF, 0.8kV) and transferred to 9.2ml pre-warmed GMEM complete. Cells were then plated in gelatinized 10cm tissue-culture dishes at 1 million cells per dish in GMEM complete. 24 hours later the appropriate selective drugs were added. Cells were cultured for a further 9-11 days, changing media every 2 days. Once colonies had reached a suitable size isolated colonies were 'picked' and transferred to gelatine-coated 96-well tissue culture plates. This was considered passage 1 for newly-derived transgenic lines. Lines were expanded by passaging to multi-well plates of increasing well size until sufficient cells were obtained to freeze down, typically a 6-well plate.

2.3.2 Transient/Episomal Transfections – Lipofection

Forced expression of transgenes in mESCs can also be achieved by transfection of intact plasmids encoding the desired transgene. Plasmids were introduced using Lipofectamine 2000 (Invitrogen, 11668-019). mES cells were harvested by trypsinisation and pipetted up and down to ensure a single cell suspension. Cells were plated in gelatinized 6-well plates at 1 million/well in GMEM complete. 3µL Lipofectamine/well was mixed in 250µL GMEM complete without serum and incubated for 5 minutes at room temperature. 3µg plasmid DNA/ml was mixed in 250µL GMEM complete without serum. The Lipofectamine and DNA mixes were combined and incubated for at least 20 minutes at room temperature. 30 minutes to 1 hour after plating the cells 500µL of Lipofectamine/DNA mix was added to each well. 24 hours later media was changed and selection added where appropriate.

2.3.3 Luciferase Assay

Luciferase assays were carried out by cotransfection of a renilla luciferase plasmid and the appropriate luciferase-reporter plasmid. The plasmids were introduced into ES cells as described for lipofection above using 4μg reporter plasmid DNA and 0.2μg renilla plasmid DNA per well. The following day cells were trypsinised and replated in 24-well plates (1 6-well can be divided into 12 24-wells) containing the appropriate media. After a further 8-24 hours' culture cells were washed once in PBS lysed on the wells in 100μL passive lysis buffer from the Promega Dual Luciferase Assay Kit and the lysates either frozen or used directly for a luciferase assay (see molecular biology methods). To assess activation of canonical Wnt signalling a TOPFlash (TCF/LEF reporter) plasmid was used. To assess STAT3 activity an acute phase response element (APRE) luciferase reporter (plasmid AGS 895) was used.

2.3.4 Making Cos7-conditioned medium – FuGene Transfections

~10^6 Cos7 cells per plate were plated onto 10cm plates 24hrs prior to transfection. Cells were transfected using FuGene (Roche, 11815091001) according to the manufacturer's protocol. GCSF-CM was generated by transfecting with plasmid pCAGGS-hGCSFb (AGS 688). Media was replaced 24hrs after transfection to prevent carry over of FuGene. After 4 days' further culture media was harvested and filtered. GCSF-CM was tested as described (Burdon et al., 1999b). See also ONPG assay.

2.3.5 Clonal Assays

To assess the ability of single mES cells to form undifferentiated colonies cells were dissociated and plated at clonal density. Media containing the appropriate cytokines was placed in gelatinized 6-well plates which were placed in the incubator to pre-equilibrate. Cells were harvested by trypsinisation and a single cell suspension ensured by pipetting up and down through a narrow-mouthed pipette. Cells were counted using a haemacytometer and diluted to a final cell density of 1000 cells/ml.

600μL of this cell suspension was added to each well to obtain a final cell number of 600/well. Cells were allowed to grow for 6-7 days before they were fixed, stained for alkaline phosphatase (see molecular biology methods), and scored for the frequency of undifferentiated (alkaline phosphatase-positive), mixed and differentiated (alkaline phosphatase-negative) colonies by microscopy.

2.3.6 Single Cell Deposition

96-well plates were gelatinized and 100μL of the desired media placed in each well. Plates were pre-equilibrated in 37C incubators prior to cell deposition. Single mES cells were deposited in individual wells of 96-well plates using a Mo-Flo cell sorter. Cells were sorted for absence of ToPro3 staining (live cells) and, where stated, for GFP expression.

2.4 Molecular Biology

2.4.1 RNA Extraction

0.5-2 million cells were harvested by trypsinisation, washed in PBS and lysed in buffer RLT (Qiagen RNeasy kit). Lysates were snap-frozen in Ethanol/dry ice and stored at -80C or used directly for RNA extraction. Lysates were homogenized by spinning through a Qiashredder (Qiagen, 79654) and subjected to RNA extraction using the RNeasy Kit (Qiagen, 74104) according to the manufacturer's protocol and incorporating an on-column DNase digest (Qiagen, 79254) to remove genomic DNA. Eluted RNA concentration was determined by spectrophotometry.

2.4.2 cDNA Synthesis

cDNA was synthesized using the Invitrogen Superscript III First-Strand Synthesis Kit (Invitrogen, 11752050) according to the manufacturer's protocol using oligo-dT

primers. 0.5-2µg RNA was used as template. cDNA was diluted 1:10 in sterile water and used for PCR and qRT-PCR.

2.4.3 RT-PCR

Routine RT-PCR was performed using Taq (Qiagen, 201203). Annealing temperatures and extension times were adjusted to optimise conditions for the given primer pair. Sequences amplified for cloning were amplified with proof-reading Phusion DNA Polymerase (Finnzymes, F540). See table for primers and conditions.

Table2.1. PCR primers.

Gene	5'-primer:	3'-primer:	Annealing Temp. (°C)
Gapdh	cccactaacatcaaatgggg	ccttccacaatgccaaagtt	55
Nanog	caggtgtttgagggtagctc	cggttcatcatggtacagtc	57
Oct3/4	gtgactgcctaccagaatga	attgtccgcataggttggag	55
Rex1	ttggggcgagctcattactt	ttgccacactctgcacacac	57
Fgf4	cacgagggacagtcttctgg	cgtcggtaaagaaaggcaca	58
Sox1	cctcggatctctggtcaagt	tacagagccggcagtcatac	55 (Use Q buffer)
T	gtgactgcctaccagaatga	attgtccgcataggttggag	56 (Add 5% DMSO)

2.4.3.1 Standard PCR

Routinely 2µl of cDNA template is amplified in a 25µl reaction volume as per Qiagen standard PCR protocol (Qiagen, 201203). Thermal cycling was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems), with general conditions as follows:

94ºC 5 minutes

30 cycles

94ºC 1 minute

XºC 1 minute

72ºC 30 seconds

72ºC 10 minutes

4ºC ∝

2.4.3.2 Blunt PCR for cloning

For cloning, 200ng of plasmid, or ES cell cDNA was used as a template for PCR in a 50µl reaction volume as per manufacturer's protocol (Phusion, High Fidelity Taq, Finnzymes, F540). Thermal cycling was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems), with general conditions as follows:

94ºC 5 minutes

30 cycles

94ºC 1 minute

XºC 1 minute

72ºC 1 minute

72ºC 10 minutes

4ºC ∝

DNA was run on an agarose gel to verify amplicon size. Clean PCR products of the correct size were used for ligation reactions.

2.4.3.3 Cloning of Bcl2

Bcl2 coding sequence was amplified from ES cell cDNA. Primers were designed to incorporate flanking Xho1 and Pac1 restriction sites.

5' primer: Xho1 Kosak
agcCTCGAGgccgccaccATGGCGCAAGCCGGGAGAAC

3' primer: Pac1
CTATTAATTAATCACTTGTGGCCCAGGTATG

2.4.3.4 *Stat3* PCR-genotyping

To test for the presence of wild-type and null *Stat3* alleles in ES cells genomic PCR was performed. The PCR mix, primers and cycle program were as follows:

Genomic DNA	5µL
10x Buffer	3µL
Primer 1 (10µM)	1µL
Primer 2 (10µM)	1µL
Primer 3 (10µM)	1µL
dNTPs	0.6µL
Taq	0.5µL
dH2O	17.9µL

Primer 1: 5'-ttgctgctctcgctgaagcgcagtagg-3'
Primer 2: 5'-cctgcttgccgaatatcatggtggaaa-3'
Primer 3: 5'-gagctgcctgaggatagaggaacctga-3'

94ºC 5 minutes
30 cycles
94ºC 15 seconds
60ºC 12 seconds
72ºC 30 seconds
72ºC 10 minutes
4ºC ∝

2.4.4 qRT-PCR

Real-time quantitative PCR was performed using the Applied Biosystems 7900 machine. Either Sybr Green or Probe-based qRT-PCR was performed.

2.4.4.1 Sybr Green Real-Time PCR

Sybr Green real-time PCR employs a dye (Sybr Green) that specifically binds double stranded (ds) DNA and fluoresces when bound. The intensity of fluorescence therefore reflects the amount of dsDNA in the reaction. Reactions were performed in 20µL reaction volumes using the Sybr Green Master Mix (Applied Biosystems, 4309155), and the standard cycler programme. For each primer/probe combination a master mix was prepared for n+1 reactions and aliquoted into 96-well reaction plates to which 2µL cDNA was added directly. See table for primers.

Master Mix (per reaction):

Sybr Green Master Mix:	10µL
Primer (F+R, 10µM):	0.4µL
PCR-grade water:	7.6µL

Table2.2. Sybr Green Real-Time PCR primers.

Gene	5'-primer:	3'-primer:
cMyc	ggaggaagccgacaacaatg	tctcaatgctagttcgctttctctt
Egr1	cagcgccttcaatcctcaag	gcgatgtcagaaaaggactctgt
Gapdh	ccctgaacggcgagatca	ttttttcctcttgcctcctgaa
Gata4	tatcacaagatgaacggcatcaa	acagcgtggtggtggtagtct
Gata6	aatgctgagggtgagcctgt	aagtggtcgaggcacccc
Id1	catgggttcgcctctcttttaa	gcagtctgcgggagagttg
Nanog	ggatgaaggcgcctgtgt	cttatgcaaggagtttaacatgatctg
nMyc	gacacgtggcaaaagaagatagtc	agtgaggcgatcctgctttc
Oct3/4	tggaaaccccgcagaca	ggtcatagttcctgttggtgaagtt
Rex1	tgtgtccgtcgtggatctga	cttcaccaccttcttgatgtcatc
Socs3	cgccatcacactgacatgagt	tcccagaattcgatgcttcct
T	gccacctggactcctatgagaa	gagcatcatactgatccaggaactc

2.4.4.2 UPL Real-Time PCR

96-well plates were used together with the fast block, Fast TaqMan Master Mix (Applied Biosystems, 4366073) and the fast protocol. Probes were obtained from the Roche Universal Probe Library (UPL) and primers designed using the Roche UPL software (https://www.roche-applied-science.com). UPL probes are labelled with FAM which fluoresces upon dissociation from the synthetic oligonucleotide. Dissociation occurs upon amplification of the target sequence allowing fluorescence levels to provide a read out of PCR product levels. Probes were used at a final concentration of 100nM and primers at 200nM. The reaction volume was 20µL. For each primer/probe combination a master mix was prepared for n+1 reactions and aliquoted into 96-well reaction plates to which 2µL cDNA was added directly. See table for primers and corresponding UPL probe numbers.

Master Mix (per reaction):

Fast TaqMan Master Mix (2x):	10µl
Primer (F+R mix, 10µM):	0.4µL
Probe:	0.2µL
PCR-grade water:	7.4µL

Applied biosystems software automatically calculates cycle threshold (CT) values from the amplification curves. Technical duplicates of each reaction were performed and the CT values averaged before calculating relative expression. Relative levels of gene expression were calculated by normalising genes of interest to *β-actin* expression and arbitrarily assigning a sample as the reference to which all other samples were compared. Errors were calculated from biological duplicate or triplicate samples.

Table2.3. UPL Real-Time PCR primers and probes.

Gene	Forward Primer	Reverse Primer	Probe
Actb	ctaaggccaaccgtgaaaag	accagaggcatacagggaca	64
Axin2	gcaggagcctcacccttc	tgccagtttctttggctctt	50
Bcl2	agtacctgaaccggcatctg	ggggccatatagttccacaaa	75
Bcl-xl	tgaccacctagagccttgga	tgttcccgtagagatccacaa	2
Cdx1	acgccctacgaatggatg	cttggttcgggtcttaccg	70
Egr1	ccctatgagcacctgaccac	tcgtttggctgggataactc	22
Fgf5	aaaacctggtgcaccctaga	catcacattcccgaattaagc	29
Gata4	ggaagacaccccaatctcg	catggccccacaattgac	13
Gata6	ggtctctacagcaagatgaatgg	ggtctctacagcaagatgaatgg	40
Klf2	ctaaaggcgcatctgcgta	tagtggcgggtaagctcgt	48
Klf4	cgggaagggagaagacact	gagttcctcacgccaacg	62
Mcl1	taacccagccatggaagttt	cagctttcatttcacccttg	33
Myc	cctagtgctgcatgaggagac	tcttcctcatcttcttgctcttc	77
Nanog	ttcttgcttacaagggtctgc	agaggaagggcgaggaga	110
nMyc	cctccggagaggataccttg	tctctacggtgaccacatcg	69
Pou5f1	gttggagaaggtggaaccaa	ctccttctgcagggctttc	95
Rex1	tcttctctcaatagagtgagtgtgc	gctttcttctgtgtgcagga	71
Socs3	atttcgcttcgggactagc	aacttgctgtgggtgaccat	83
T	cagcccacctactggctcta	gagcctggggtgatggta	100
Tbx3	ttgcaaagggttttcgagac	tgcagtgtgagctgctttct	51
Tcl1	cgtgtacttggatgagtttcgt	gcaagatcacctggaatttttc	71
Zfx	accgtccggtgcgtataa	ttctcatcagccagaacacct	104

2.4.5 Agarose Gel Electrophoresis

Agarose gels were prepared by melting agarose at the desired concentration (1-2%) in TBE buffer. Molten agarose was cooled, Sybr Safe nucleic acid dye (Invitrogen, S33102) added and gels poured in casting trays. Electrophoresis was performed in

Mupid gel tanks at 25-130V in 0.5x TBE buffer. Nucleic acid was visualized by UV transillumination and pictures taken using the G-box.

2.4.6 Gel Extraction

Bands were purified from agarose gels by cutting bands of the desired size on a transilluminator and purifying nucleic acids using the Qiagen Gel Extraction Kit (Qiagen, 28704) according to the manufacturer's protocol. The concentration of purified DNA was determined by spectrophotometry. DNA fragments were used in subsequent ligations.

2.4.7 Spectrophotometry

Concentrations of nucleic acid were measured using a Nanodrop spectrophotometer.

2.4.8 TOPO-Cloning

PCR products were cloned into TOPO vector using the Zero Blunt TOP PCR cloning kit (Invitrogen, K2800-20) for blunt ended PCR products or the TOPO TA Cloning Kit (Invitrogen, KNM4600-01) for PCR products with a polyA overhang. Colonies formed on ampicillin plates were miniprepped and test digested with EcoR1 to check for insertion of the PCR product. Minipreps carrying the insert were sequenced from M13F and M13R primers and the sequence checked for mutations before proceeding with subcloning reactions.

2.4.9 Ligations

Ligations were carried out using Quick Ligase (NEB, M2200S) according to the manufacturer's protocol. Ligation products were transformed into competent cells, plated on selective agar plates, colonies picked and miniprepped. Miniprepped DNA

was checked for successful ligation by restriction digest and gel electrophoresis before use in subsequent experiments.

2.4.10 Restriction Digests and Ethanol Precipitation

All enzymes were obtained from New England Biolabs and used according to the manufacturer's protocol.

For stable transfections plasmid DNA was linearised by digestion with Sca1.

DNA was purified from digest reactions by precipitation. 2.5 volumes 100% EtOH and 1/10 volume 3M Na Acetate were added to the reaction and precipitated DNA pelleted by centrifugation at maximum speed for 10mins in a microcentrifuge. Supernatant was removed and the pellet washed in 70% EtOH followed by a further 10mins centrifugation. The supernatant was removed, the pellet dried for 10-15mins and redissolved in dH2O or TE buffer.

2.4.11 Transformations

Plasmid DNA was transformed into competent *E. Coli* (DH5α). 50µL cells were thawed on ice, incubated on ice with 1µL plasmid DNA for 20 minutes, heat shocked for 30 seconds at 37C, placed on ice for 2 minutes, 1ml LB added and tubes shaken at 37C for 1 hour before plating 10 or 100µL on the appropriate selective plates. Plates were incubated overnight at 37C.

2.4.12 Plasmid Purification

Single bacterial colonies were picked from fresh plates into the required volume of LB containing the appropriate selection (100µg/ml ampicillin or 50µg/ml kanamycin) and shaken overnight at 37C. Plasmid DNA was isolated by Miniprep

(Qiagen, 27106) or Maxiprep (Qiagen, 12162) according to the manufacturer's protocol.

2.4.13 Plasmids

AGS 796: pCAGgrgpY118FIZ

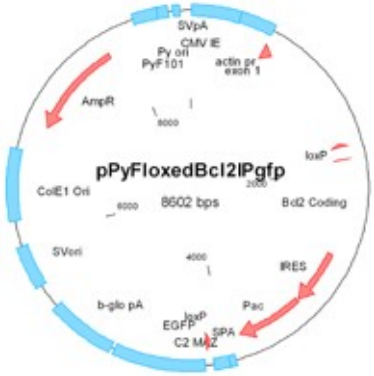
AGS 1126: pPy Floxed MT IP gfp

AGS 1233: pPyCAG KLF4 IH

pCAG Cre IRES GFP: Gift from Sandra Gomez Lopez

pPy Floxed Bcl2 IP gfp.

PCR-amplified Bcl2 coding sequence was cloned into TOPO vector and verified by sequencing. TOPO-Bcl2 and AGS 1126 were digested with Xho1 and Pac1 and fragments of the appropriate size isolated from agarose gels. Bcl2 coding sequence was then ligated into AGS 1126 to form pPy Floxed Bcl2 IP gfp. Cells expressing this construct can be reverted by exposure to Cre and sorting of GFP+ cells. Cre recombinase recombines flox sites flanking the coding sequence and puromycin resistance genes bringing GFP under control of the CAG promoter.



2.4.14 Preparation of genomic DNA

Genomic DNA was prepared using the DNeasy Blood and Tissue Kit (Qiagen, 69504). At least 1 million cells were harvested by trypsinisation, washed in PBS and genomic DNA extracted according to the manufacturer's protocol. Genomic DNA was used directly for PCR genotyping.

2.4.15 Western Blotting

2.4.15.1 Solutions and reagents

Laemmli Lysis Buffer:	2% SDS 10% Glycerol 60mM Tris pH6.8 0.1M DTT Bromophenol blue
Running Buffer:	NuPage MOPS, 20x (Invitrogen, NP0001)
Antioxidant:	NuPage Antioxidant (Invitrogen, NP0005)
Transfer Buffer:	20% MeOH 25mM TRIS 192mM Glycine In dH2O
TBS:	10mM TRIS 150mM NaCl In dH2O pH7.6-8.0
Blocking Buffer:	10% dried, skimmed milk (Marvel) 0.05% NP40 In TBS

Incubation Buffer:	5% dried, skimmed milk (Marvel) 0.15% NP40 In TBS
Wash Buffer:	10mM TRIS 650mM NaCl 0.3% Triton x100 In dH2O pH7.6-8.0
Stripping Buffer:	2% SDS 62.5mM TRIS pH6.8
Gels:	10% NuPage Bis-Tris (Invitrogen, NP0301BOX) 12-14% NuPage Bis-Tris (Invitrogen, NP0321BOX)
MW marker:	SeeBlue Plus2 (Invitrogen, LC5925)
Gel-tank:	Novex Mini-Cell (Invitrogen, EI0001)
Membrane:	Nitrocellulose (Hybond-ECL, Amersham, RPN203D)
ECL:	ECL-plus (Amersham, RPN2132)

2.4.15.2 Sample Preparation

~2 million cells were lysed directly on the wells of 6-well plates in 250µL laemmli lysis buffer and transferred immediately to eppendorf tubes on ice. The samples were boiled for 5 minutes, sonicated briefly and subjected to SDS-PAGE directly or stored at -20C.

2.4.15.3 Running the Gel

10% or 12-14% Novex gels (Invitrogen) were used. The comb was removed, wells rinsed by pippetting running buffer into the wells and 20µL sample loaded per well. 10µL protein standard was loaded in 1 well. Gels were run at 200V for approximately 1hr.

2.4.15.4 Transfer

The gel was removed from its casing into transfer buffer, the foot and wells removed, and assembled with the membrane between 1 sponge and 2 sheets wet Wattman paper either side, taking care to avoid bubbles. The assembly was transferred at 395 amps for 70 minutes in a cold room in BioRad gel tanks.

2.4.15.5 Blocking, antibody incubation, washing and stripping

The membrane was placed protein side up in a 50ml tube and incubated for 2hrs in blocking solution. Primary antibodies were diluted in 5ml 5% milk and incubated with the membrane for 2hrs at RT or overnight at 4C. Secondary antibodies were diluted in 5% milk and incubated with the membrane for 1hr at RT. Following primary and secondary antibody incubations membranes were washed 3 x 15 minutes in 30ml wash buffer. Membranes were incubated for 5 minutes with ECL, exposed to X-ray film and developed. If membranes were to be reprobed antibodies were stripped by incubating for 30 minutes at 70C in stripping buffer. Following stripping membranes were re-blocked. See table for details of antibodies and concentrations.

Table2.4. Antibodies for immunoblotting and immunofluorescence (IF)

	Antibody	**Source**	**Cat. No.**	**Dilution (1/)**
Primary	β-catenin	BD Transduction Laboratories	610153	1000
	p-β-catenin(ser33/37/thr41)	Cell signalling	9561	1000
	p44/42 MAPK (ERK)	Cell signalling	9102	1000
	p-p44/42 MAPK(thr202/tyr204)	Cell signalling	9101	1000

	AKT (PKB)	R and D systems	MAB2055	1000
	p-AKT(ser473)	R and D systems	AF887	1000
	p38	Cell Signalling	9212	1000
	p-p38(thr180/tyr182)	Cell signalling	9211	500
	Nanog	Abcam	ab21603	200 (IF)
	Oct3/4 (c10)	Santa Cruz Biotechnology	sc-5279	200 (IF) 500
	Gata4 (c-20)	Santa Cruz Biotechnology	sc-1237	100 (IF)
	Myc	Santa Cruz Biotechnology	sc-42	200
	NRDG1	Cohen laboratory		1µg/ml
	p-NRDG1	Cohen laboratory		1µg/ml
	Alpha-Tubulin	Abcam	Ab7291	5000
	Tuj1	Covance	MMS-435P	400 (IF)
	Stat3	BD Transduction Laboratories	610189	1000
	pStat3 (Tyr705)	Cell Signaling Technology	9131	1000
	GSK3-α/β	Biosource	44-610	1000
	Id1 (c-20)	Santa Cruz Biotechnology	sc-488	1000
	Nestin	DSHB		50 (IF)
	Blbp	N. Heins		500 (IF)
	Rc2	DSHB		50 (IF)
	Sox2	Chemicon	AB5603	1000 (IF)
Secondary	ECL Mouse IgG, HRP-Linked Whole Ab	Amersham	NA931	2000-5000
	ECL Rabbit IgG, HRP-Linked Whole Ab	Amersham	NA934	2000-5000
	Alexafluor (for IF)	Alexa		500-1000 (IF)

2.4.16 Immunoprecipitation

2.4.16.1 Buffers and Reagents

IPH BUFFER (as used in (Frank et al., 2003) for immunoprecipitation of cMyc from whole cell lysates): 50 mM Tris-HCl pH 8

150 mM NaCl

5mM EDTA

0.5% NP40

1 mM DTT before use

One tablet of protease inhibitor before use

A/G sepharose beads (Amersham)

2.4.16.2 Sample Preparation

mES cells were grown in 10cm dishes in the appropriate conditions. Cells were washed once in cold PBS and 1ml cold IPH buffer added directly to the plates. The plates were rocked at 4C for 30mins, lysates collected by scraping the plates with a cell scraper (Greiner) and pippeting the lysate into 1.5ml eppendorfs on ice. Lysates were spun in a microcentrifuge at 13000rpm at 4C for 1 minute and supernatants transferred to clean 1.5ml eppendorfs on ice. At this point 50µL lysate was removed from each sample, mixed with 2x laemmli buffer (see western blotting), and boiled for 5mins. These were input samples used as loading controls.

2.4.16.3 Immunoprecipitation (IP)

20µL protein A sepharose bead slurry per sample was placed in 1.5ml eppendorfs, centrifuged, supernatant removed and beads washed in 0.5ml IPH buffer 2-3 times. 2µg appropriate antibody was added per sample and cleared lysate (see sample preparation) added to the beads and antibody. The tubes were tumbled for 4-6hrs at 4C, beads pelleted by centrifugation, supernatant removed and beads washed in 1ml cold IPH buffer 5 times. On the last wash care was taken to remove all supernatant, 30µL 2x Laemmli Buffer was added to the pelleted beads and samples were boiled for 5mins. Lysates were stored at -20C or used directly for SDS-PAGE. Samples were processed as for western blotting.

2.4.16.4 IP Controls

MOCK: IP performed with alternative antibody, same isotype. In the case of cMyc IP anti-tubulin was used as a control.

Bead Only: IP performed without antibody. Controls for non-specific binding to beads.
No lysate: IP performed with lysis buffer instead of cell lysates. Allows immunoglobulin bands to be unambiguously identified on immunoblots.

2.4.17 Alkaline Phosphatase Staining

Fixative: 65% Acetone
25% Citrate (Solution in kit)
8% Formaldehyde

Stain: From Alkaline Phosphatase (AP) kit (Sigma 86R-1KT) according to manufacturer's protocol

Adherent mES cells were washed once in PBS and fixed for 45 seconds in fixative. Plates were then washed in dH2O and alkaline phosphatase stain added. Plates were incubated with the stain for 30 mins in the dark, washed in dH2O and dried. Stained colonies were scored by eye using a dissecting microscope. Colonies are classified as undifferentiated (AP-positive), mixed or differentiated (AP-negative).

2.4.18 Immunofluorescence

Expression of particular proteins in adherent cells was detected by immunology. Cells were exposed to antibodies recognising the protein of interest and subsequently to fluorescently labelled secondary antibodies (Alexa). Staining was analysed by fluorescence microscopy.

Cells were fixed in 4% PFA for 15 minutes, blocked and permeablised in PBS, 0.1% Triton x100, 3% Goat/Donkey Serum, 1% BSA. Primary antibodies were incubated in the same buffer overnight at 4C. Secondary antibodies were incubated for 1hr at

RT. Plates were washed 3 x 15mins in PBS after primary and secondary antibody incubations. Nuclei were stained with DAPI.

2.4.19 ONPG Assay

Lysis Buffer: 0.25 M Tris
pH 7.5
0.5 mM DTT
0.5% NP-40

ONPG Buffer: 60 mM Na2HPO4
40 mM NaH2PO4
10 mM KCl
1 mM MgCl2
50 mM 2-mercaptoethanol
1.2 mM ONPG

Expression of β-galactosidase from the *Oct4* locus in D027 cells was quantitated in an ONPG assay as described (Burdon et al., 1999b). ES cells were plated at 5000 cells per well in 24-well dishes and cultured for 6 days in the presence or varying concentrations of LIF or GCSF-conditioned medium. On day 6, cells were washed once with PBS and lysed in 0.4 ml of lysis buffer. Lysate (40µl) was mixed with 100µl of ONPG buffer in a microtitre plate and incubated at 37°C for 2–4 hrs and the absorbance was read at 420 nm. All assays were performed in triplicate.

2.4.20 Luciferase Assay

Lysates were prepared as described in section 2.3.3. Luminescence was assessed using the dual luciferase reporter assay system (Promega, E1910). 10µl lysate was pipetted into a well of a 96-well plate. Automated injection and luminescence

readings were performed on a GloMax luminometer (Promega). The luminometer was programmed as follows.

Injector 1, LARII: 50µl, 2 sec lag, 10 sec read
Injector 2, Stop and Glo: 50µl, 2 sec lag, 10 sec read

Final values were calculated as the ratio of luciferase to renilla luminescence. Values were averaged for 3 biological replicates.

2.4.21 Flow Cytometry

2.4.21.1 Apoptosis Assay

ES cells cultured in the conditions of interest were harvested by trypsinisation. The proportion of apoptotic cells in the population was measured using the AnnexinV Apoptosis Kit (BD Biosciences, 556547) according to the manufacturer's protocol. Fluorescence was analysed by flow cytometry using a Cyan (DAKO). Apoptotic cells were defined as those cells that were PI negative and AnnexinV positive. Unstained cells served as a negative population for calibration. As a positive control apoptosis was induced by 4hrs incubation with 4µM camptothecin (Sigma, C9911). Annexin V only- and PI only-stained cells were used to compensate for 'bleed through' between the FITC and PE channels.

2.4.21.2 Sorting of GFP-positive and -negative cells

ES cells expressing GFP were separated from GFP-negative cells by flow cytometry using a MoFlo (DAKO). Wild-type cells were used to set the parameters for identification of GFP-positive cells. Dead cells were excluded by sorting for ToPro3-negative cells. GFP-positive and negative cells were collected in the appropriate media in separate tubes, counted and plated as required.

Chapter 3: Analysis of intracellular signalling pathways and inhibitor specificity in three inhibitors (3i)

3.1 Introduction

Defined conditions for mouse ES cell culture and derivation were established in 2003 (Ying et al., 2003a). A serum-free media composition containing the supplements N2 and B27, known as N2B27 (Ying and Smith, 2003), was used. Addition of the cytokines LIF and BMP4 was sufficient for *de novo* derivation and clonal propagation of ES cells. If BMP4 is removed from the media the cells will eventually undergo neural differentiation while exposure to BMP4 alone results in differentiation into large, flattened cells of a non-neural lineage (Ying et al., 2003a). The molecular mechanisms underlying the effects of LIF and BMP4 have been investigated and the key downstream signalling molecules identified (Niwa et al., 1998; Burdon et al., 1999b; Matsuda et al., 1999; Ying et al., 2003a). However, it has not been possible to derive ES cells from other species using these growth conditions and LIF does not support the culture of established hES cells (Thomson et al., 1998; Daheron et al., 2004; Humphrey et al., 2004). It is therefore desirable to develop alternative culture conditions that will support ES cell derivation from a range of mammalian species, demonstrating cross-species commonalities in ES cell biology, if they exist, and allowing researchers to extend their findings from the mouse model to rat, human and beyond. To this end our group undertook an examination of the effect of several well known pharmacological inhibitors on the self-renewal of mouse ES cells.

3.1.1 Rationale for the study

As discussed in the introduction, many signalling pathways have been implicated in the regulation of self-renewal on mES cells. Although fully defined conditions for derivation and culture had been established their application was limited to 129 strain

mice (Ying et al., 2003a) and had not been successfully applied to any other species. The possibility of developing culture conditions based on previous studies which have demonstrated that the manipulation of other signalling pathways can influence the efficiency of self-renewal (Burdon et al., 1999b; Sato et al., 2004) was attractive to further our understanding of pluripotency and to extend ES cell technology beyond 129 mice.

Inhibition of ERK1/2 signalling has been shown to promote self-renewal in several previous studies (Burdon et al., 1999b; Buehr and Smith, 2003). In fact, through use of a MEK inhibitor ES cells were derived from CBA strain mice (Buehr and Smith, 2003) without the need for the technically demanding technique of microdissection (Brook and Gardner, 1997). Signalling through ERK1/2 can be activated by cytokines and growth factors including LIF, FGFs and insulin (reviewed in (Katz et al., 2007)). It has been shown that altering the balance of LIF signalling in favour of Stat3 over ERK1/2 promotes self-renewal (Burdon et al., 1999b; Forrai et al., 2005) and pharmacological inhibition of MEK1/2 inhibits differentiation in EBs (Burdon et al., 1999b). ES cells express FGF4 and its deletion decreases differentiation in colony forming assays (Wilder et al., 1997) indicating that autoinduction of FGF signalling promotes differentiation. The MEK-ERK signalling cascade is a major downstream pathway of FGF signalling and given what is known about the involvement of ERK1/2 signalling in self-renewal it seems likely that this pathway mediates the effect of FGF4. Blockade of the FGF4-MEK-ERK1/2 cascade became a focus in our investigations.

Both canonical Wnt signalling and direct inhibition of GSK3 have been used to manipulate the *in vitro* culture of ES cells (Sato et al., 2004; Liu et al., 2006; Ogawa et al., 2006). The identification of a novel GSK3 inhibitor named BIO and the demonstration that it could effect a short-term self-renewal benefit on the culture of m- and hES cells (Sato et al., 2004) has been at the centre of investigations largely focused on the activation of β-catenin and its role in self-renewal. Although the various studies in the literature fail to agree on the sufficiency of otherwise of β-catenin activation for self-renewal and all of the studies to date rely on undefined

conditions and often the poorly controlled use of pharmacological inhibitors and conditioned media there is more than enough evidence to justify an in-depth investigation of the role of Wnt signalling and GSK3 in the regulation of self-renewal.

3.1.2 Pharmacological inhibitors in the study of signalling

Signalling pathways can be activated by the provision of cytokines or growth factors in the culture media. The limitations of this become clear when considering the complexity of signalling. Any given growth factor might activate more that one molecular pathway and different signalling molecules can activate the same pathway. Furthermore, the output of a given pathway is influenced by the context in which it is activated; pathways may converge on targets and act synergistically or they may cross-antagonise one another. Therefore, in order to dissect the components of signalling pathways biochemical and genetic tools must be employed. Pharmacological inhibitors are designed to target specific molecules to inhibit their function. Inhibition of a pathway provides a complementary approach to its activation and as the targets of an inhibitor can be downstream of a receptor and specific to a given signalling pathway they allow the different signalling arms to be isolated and analysed for their effects. Inhibitors can also activate signalling if their target molecules are themselves inhibitors, as is the case for GSK3 (Doble and Woodgett, 2003). Caution must be exercised when employing inhibitors and interpreting their effects because the inhibitors often interact with molecules not intended as their targets (Bain et al., 2007), particularly at high concentrations, and it is impossible to know the effect of an inhibitor on all the signalling molecules present in a cell. It is desirable therefore to use inhibitors that have been extensively characterised with regards to their specificity and where possible controls should be provided for the effect of an inhibitor by employing alternative inhibitors of the same pathway and genetic controls where the target molecule has been deleted (Bain et al., 2007). Genetic manipulations are another means of studying signalling. They provide an advantage over inhibitor studies because, when the appropriate rescue experiments are performed, the effect can be reliably attributed to a specific target

molecule. The disadvantage is the technically demanding and time consuming aspect of genetically modifying cells and organisms. The use of inhibitors also allows studies to be carried out in genetically unmodified cells, an important consideration when working with the derivation and culture of ES cells. Thus, inhibitor studies and genetic manipulations provide complementary methods for the analysis of signalling pathways.

3.1.3 Inhibitors of the FGF-MEK-ERK1/2 signalling pathway

The FGF family consists of 22 ligands and 4 receptors (reviewed in (Eswarakumar et al., 2005)). ES cells express FGFR-1, -2 and -4 (Chen et al., 2000) and the ligand FGF4 (Rathjen et al., 1990; Ma et al., 1992). FGFRs are receptor tyrosine kinases (RTKs) and autophosphorylate tyrosine residues on their intracellular domains when ligand-bound. Phospho-tyrosine residues act as docking sites to recruit SH2-domain containing proteins. Recruitment of phospholipase C (PLC) results in its activation and downstream signalling activates Ca^{2+} release. The PI3K pathway is also activated through the recruitment of Gab1. Through Grb2 the Ras-Raf-MAPK pathway is activated resulting in phosphorylation and activation of ERK1/2 (reviewed in (Eswarakumar et al., 2005)). Inhibition of the FGFR is predicted to block activation of all of these downstream signalling pathways. In this study two structurally distinct FGFR inhibitors have been used (Fig3.1). A class of compounds, pyrido[2,3-*d*]pyrimidines, were identified in a screen for inhibitors of FGF and PDGF receptors (Connolly, 1997). Modifications of this core identified PD173074 (PD17), a specific inhibitor of the FGFR (Mohammadi et al., 1998). In another screen of chemicals, generated by attaching different chemical substituents to an oxindole core, SU5402 (SU) was identified as an inhibitor of FGFR1 (Mohammadi et al., 1997). Both inhibitors block autophosphorylation of the receptor in response to ligand binding. SU has an IC50 (the inhibitor concentration at which target activity is reduced by 50%) of 10-20μM while PD17 is more potent with an IC50 of around 25nM. Both are reported to inhibit PDGFR at much higher concentrations while PD17 inhibits VEGFR at concentrations of 100-200nM. These compounds were used in parallel to assess the specificity of the effect for inhibition of the FGFR

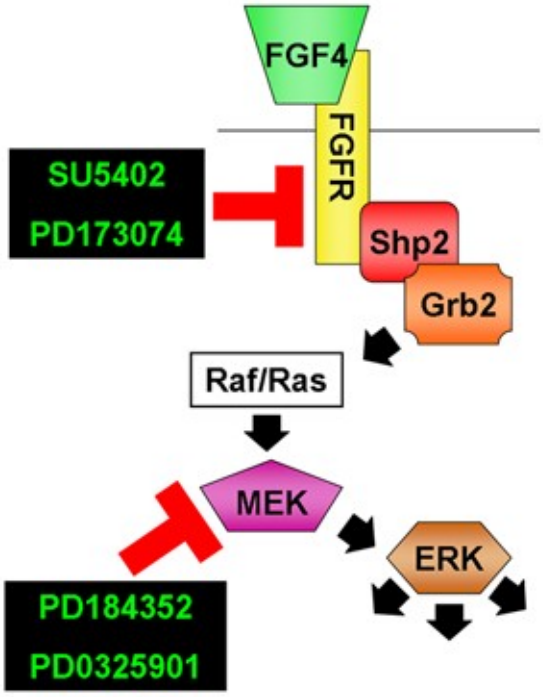


Fig3.1. FGF4-MEK-ERK Signalling and inhibitors. FGF4 binds and activates FGFRs which couple through Shp2 and Grb2 to activate Raf/Ras. MEK is phosphorylated and activated by Ras and in turn phosphorylates ERK. ERK mediates multiple downstream effects. The inhibitors SU5402 and PD173074 inhibit the FGFR while PD184352 and PD0325901 inhibit MEK. All of these inhibitors decrease activity of the downstream targets ERK-1 and -2.

To specifically block activation of ERK1/2 inhibitors of the kinases responsible for their phosphorylation and activation, MEK1/2, were employed (Fig3.1). A variety of MEK inhibitors are commercially available, U0126 and PD98059 (PD98) having been used extensively in the past. PD98 was identified in an *in vitro* compound library screen for inhibitors of MEK but not ERK itself (Dudley et al., 1995). PD98 was found to inhibit phosphorylation of the downstream ERK target, MBP, only when added to the *in vitro* system prior to the addition of ERK. This demonstrated that its effect was on the upstream kinase, MEK. Further investigation showed that PD98 bound to inactive MEK and prevented its activation by Raf (Alessi et al., 1995). The inhibitor was found to be specific by its lack of activity against a small panel of *in vitro* kinases and against MEK homologues *in vivo*. PD184352 (PD18) and PD0325901 (PD03) are derivatives of PD98 and were chosen for this study as both are reported to be more potent and more specific inhibitors of ERK1/2 than U0126 or PD98 (reviewed in(Bain et al., 2007)). PD18 is reported to almost completely inhibit phosphorylation of ERK1/2 in response to EGF (as judged by the absence of detectable phosphorylated ERK1/2 when assessed by immunoblotting) at a concentration of 0.5μM, PD03 having a similar effect at just 25nM. Both have been

tested against a panel of more than 70 kinases for off-target effects revealing that even at higher concentrations none were significantly affected with the exception of MEK5. MEK5 was inhibited by PD18 at concentrations of 10-20μM and by PD03 at concentrations over 2μM. The kinase panel is by no means exhaustive with more than 500 kinases thought to be encoded in the human genome but it goes some way towards demonstrating specificity revealing that some widely used compounds have significant off-target effects and cannot be used reliably in signalling analysis (Bain et al., 2007).

3.1.4 Inhibitors of GSK3

GSK3 has two isoforms, α and β. They are highly homologous but differ in their C-terminal domains. Both isoforms are thought to be subject to the same regulatory pathways and appear to carry out largely the same functions. However, deletion of GSK3β is lethal and embryos die at around day 16 indicating that GSK3α cannot fully rescue the loss (Hoeflich et al., 2000). GSK3 has many molecular targets and is subject to regulation by multiple signalling pathways. Its cellular functions include regulation of glycogen metabolism, cell cycle and proliferation. The implication of GSK3 in human diseases including neurological disorders, cancer, bipolar disorder and non-insulin-dependent diabetes mellitus has created a great deal of interest in the development of drugs to manipulate its function. However the fact that GSK3 is involved in so many cellular processes also means that its inhibition is likely to have effects beyond those intended therapeutically (Doble and Woodgett, 2003).

GSK3 is well known as a component of the canonical Wnt signalling pathway. It is a component of the β-catenin destruction complex which also includes Axin, a scaffolding protein, adenomatous polyposis coli (APC) and β-catenin itself. In this complex GSK3 is constitutively active and phosphorylates β-catenin on a series of serine residues, targeting it for ubiquitin-dependent proteolysis. In the presence of Wnt ligands signalling through the receptor Frizzled and Dishevelled leads to inactivation of GSK3 through a poorly defined mechanism. As part of the insulin signalling pathway GSK3 phosphorylates and inactivates glycogen synthase. Insulin

signals through PI3K to activate PKB which in turn phosphorylates GSK3 on Ser9/21 (α/β) (Cross et al., 1995), inactivating it and allowing glycogen synthase to become dephosphorylated. These functions of GSK3 are apparently independent since signalling through PI3K does not affect β-catenin phosphorylation and Wnt signalling does not affect the insulin pathway. This is probably achieved by sequestering a proportion of GSK3 in the destruction complex. Inhibitors of GSK3 are predicted to affect both pools mimicking canonical Wnt signalling and insulin signalling simultaneously. There are many known GSK3 targets not mentioned here which will also be affected by GSK3 inhibition (reviewed in (Doble and Woodgett, 2003)).

LiCl is the best known of the GSK3 inhibitors but relatively high concentrations are required and non-specific effects have been reported (reviewed in (Bain et al., 2007)). Alternative small molecule inhibitors have been developed including the compounds SB216763 (SB21) and SB415286 (SB41) (Smith et al., 2001) which have been tested as part of our study. Both have been tested against a panel of kinases and have been seen to affect a range of other kinases (Bain et al., 2007). In contrast the inhibitor CHIR99021 (CHIR, also known as CT99021) (Cline et al., 2002), when tested against the same panel of kinases was shown to have no significant effect on any of the kinases tested with the exception of CDK2-CyclinA which was inhibited with 50-fold lower potency than GSK3 itself. As CHIR is the best characterised of the available GSK3 inhibitors and is recommended as a GSK3 inhibitor by a group with expertise in GSK3 function and regulation this has been our inhibitor of choice for GSK3. More recently a novel GSK3 inhibitor has been identified and employed in the study of ES cell self-renewal (Sato et al., 2004). However questions have been raised over its specificity as it appears to interact with the FGFR (Zhen et al., 2007) and to activate Stat3 through an unknown mechanism (Ogawa et al., 2006). The lack of a thorough study on the specificity of this compound discouraged us from including it in our own experiments.

3.1.5 Three inhibitors (3i)

Using existing ES cell lines, conditions were established that supported robust self-renewal and clonal propagation. Using N2B27 (Ying and Smith, 2003) as a base inhibitors were added at the following concentrations: PD184352 (Davies et al., 2000), 0.8μM; SU5402 (Mohammadi et al., 1997), 2μM; CHIR99021(Cline et al., 2002), 3μM. This combination of three inhibitors was termed '3i'. It was tested for its ability to support derivation *de novo* and lines were readily established from 129 background mice (Ying et al, in press). After a period in culture these ES cells were tested for their ability to contribute to the embryo by injecting them into host blastocysts. High contribution was observed as judged by extensive coat-colour chimaerism and contribution to the germ line was confirmed by crossing chimaeras and observing transmission of the ES cell genome to the offspring (Ying et al, in press). This work provided the platform for analysis of these novel culture conditions and the starting point for this thesis.

3.2 Results

3.2.1 3i Supports robust self-renewal of mouse embryonic stem cells

ES cells grown in 3i form colonies of tightly packed, rounded up cells that exhibit reduced adhesion to gelatine-coated culture dishes. There is little or no evidence of spontaneous differentiation as judged by the absence of morphologically differentiated cells (Fig3.2a). 3i-cultured cells are readily distinguished from those in LIF and BMP4 or in serum and LIF which tend to grow in more flattened monolayer cultures. An important property of self-renewal is the ability of single cells to give rise to undifferentiated colonies (Smith, 2001). To test this rigorously for cells grown in 3i single cells were plated in individual wells of 96-well plates using a MoFlo cell sorter. These were incubated for 10-11 days and the number of ES cell colonies formed scored according to expression of GFP under control of the promoter of the pluripotency marker Oct4 (Ying et al., 2002) and/or colony morphology (Fig3.2b). 3i conditions supported colony formation with efficiency similar to that observed for

LIF and BMP4 (Fig3.2c,d), established ES cell culture conditions (Ying et al., 2003a).

Marker analysis of ES cells grown in 3i confirms that they express pluripotency-associated genes *Oct4, Nanog* and *Rex1* but do not express detectable levels of the differentiation associated genes *Sox1* and *Brachyury* (Fig3.2e).

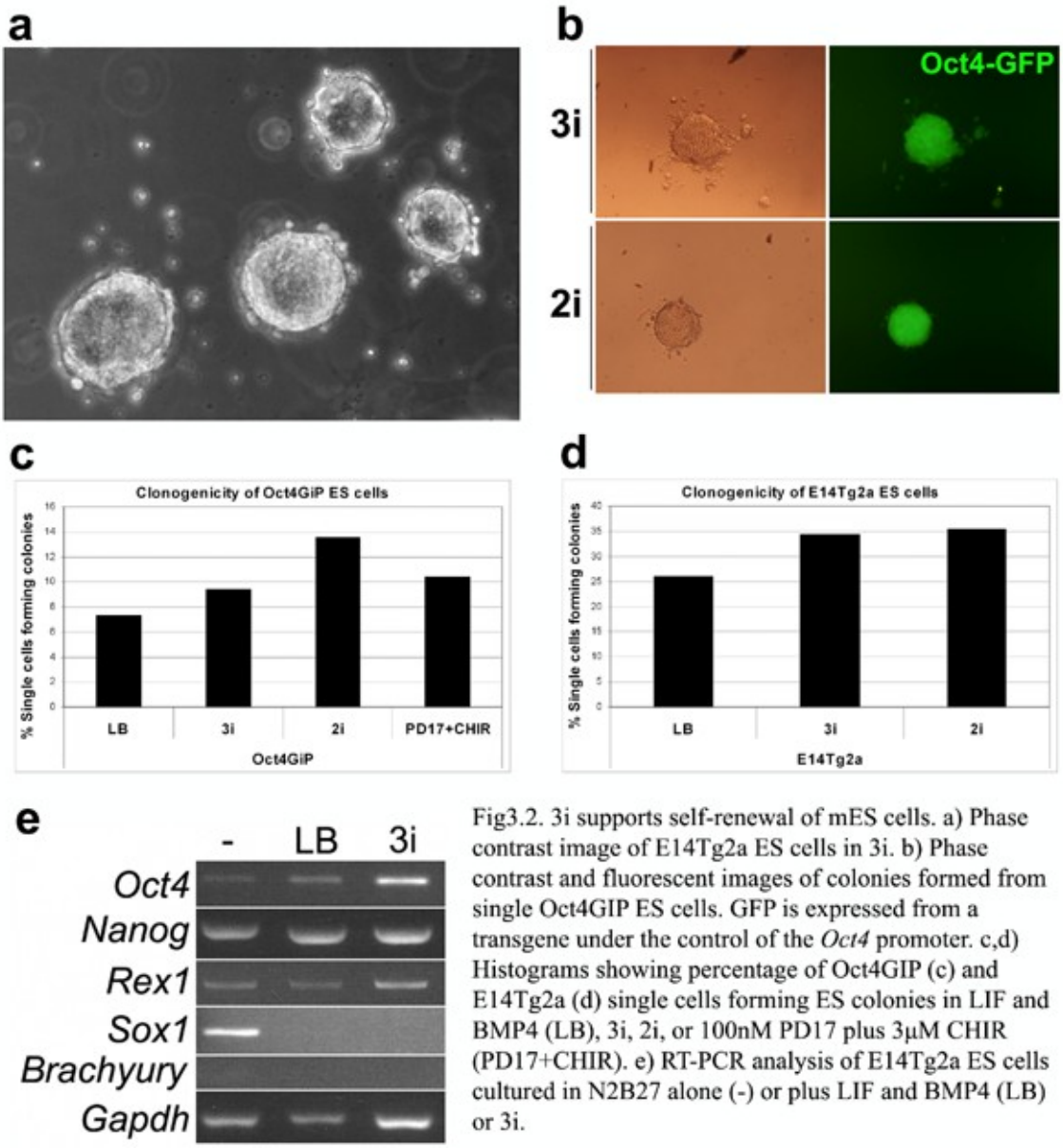


Fig3.2. 3i supports self-renewal of mES cells. a) Phase contrast image of E14Tg2a ES cells in 3i. b) Phase contrast and fluorescent images of colonies formed from single Oct4GIP ES cells. GFP is expressed from a transgene under the control of the *Oct4* promoter. c,d) Histograms showing percentage of Oct4GIP (c) and E14Tg2a (d) single cells forming ES colonies in LIF and BMP4 (LB), 3i, 2i, or 100nM PD17 plus 3μM CHIR (PD17+CHIR). e) RT-PCR analysis of E14Tg2a ES cells cultured in N2B27 alone (-) or plus LIF and BMP4 (LB) or 3i.

3.2.2 Analysis of the major signalling pathways in 3i

As discussed in the general introduction many signalling pathways have confirmed or proposed roles in the regulation of self-renewal. Here I investigate the activation status of these signalling pathways in 3i as compared to other culture conditions. All of the experiments are performed on cells grown for at least 24 hours in the indicated conditions and are therefore considered to be steady-state measurements of the activation status of the gene or protein in question. This should be the most relevant analysis when considering the effect of a pathway on the long-term culture of ES cells as opposed to acute responses that occur immediately following changes in the culture environment.

As expected, the level of phospho-ERK (pERK) was reduced by addition of either SU or PD18 (Fig3.3a). Interestingly, their combined effects (the combination of PD18 and SU is referred to as 'PS') at concentrations optimised for cell culture appeared to be synergistic. While either inhibitor used alone resulted in only a slight reduction in pERK, together they significantly reduced pERK to levels just detectable by immunoblotting. Thus, combining the inhibitors had allowed their concentrations to be reduced which should reduce off-target effects and toxicity of the compounds. Note that CHIR had no effect on ERK phosphorylation. These results also show that ES cells cultured in N2B27 alone have high levels of ERK activity indicating that ERK can be activated independently of added cytokines and growth factors or factors contained in serum. This is attributable to production of FGF4 by the ES cells themselves and activation of the FGFR and downstream MAPK signalling (Fig3.4c) (Kunath et al., 2007).

Inhibition of GSK3 is predicted to decrease levels of phosphorylation on a range of well characterised targets. Accordingly, I found that western-blotting using an antibody raised against NDRG1 (Murray et al., 2004) showed loss of the phosphorylated form as indicated by an increase in electrophoretic mobility and loss of the slower migrating band (Fig3.3b). Importantly, neither the presence of LIF and BMP4 nor PS affected NDRG1 phosphorylation. Treatment with CHIR also reduced

the phosphorylation of β-catenin. However, no significant increase in the levels of total β-catenin could be detected (Fig3.3c) as might have been predicted since the loss of GSK3-mediated phosphorylation stabilises β-catenin (Doble et al., 2007). This is likely to reflect the fact that a large proportion of cellular β-catenin is incorporated into adherens junctions (reviewed in (Bienz, 2005)) and the increase in β-catenin caused by GSK3 inhibition does not reflect a significant increase in total β-catenin. The stabilisation and nuclear accumulation of β-catenin can be tested by assaying the activation of the TCF/LEF-sensitive luciferase reporter, TOPFlash. β-catenin accumulating in the nucleus interacts with TCF/LEF TFs to activate transcription and this can be clearly seen in the dose-dependent response of the TCF/LEF reporter to CHIR (Fig3.3d). Note that 3μM CHIR, the concentration used in 3i, does not maximally activate the TCF/LEF reporter indicating incomplete inhibition of GSK3. Given that the CHIR concentration was optimised for ES cell propagation this implies that optimal self-renewal may occur when GSK3 is only partially inhibited.

The PI3K signalling pathway is an important cell survival/proliferation signal (Takahashi et al., 2005) and has also been implicated in the inhibition of differentiation in m- and hES cells (Paling et al., 2004; Watanabe et al., 2006). PKB is phosphorylated in a PI3K-dependent manner on Ser473 (Alessi et al., 1996). An antibody that specifically recognises this phosphorylated form can therefore be used as an indicator of PI3K activity. Little variation was observed in the degree of steady-state pPKB indicating that the inhibitors did not influence the level of PI3K activity in this context (Fig3.3e). The high levels of basal PI3K phosphorylation can be attributed to the inclusion of insulin in the media as a component of N2 and B27. Removal of insulin and B27 from the media results in a severe reduction in pPKB and reveals that in this context the PI3K pathway becomes responsive to the MEK inhibitor PD03 (Fig3.3f). The increase in pPKB phosphorylation is likely to result from a loss of negative feedback signalling from activated ERK which inhibits activation of the PI3K and MAPK pathways downstream of FGFRs (Lax et al., 2002) (see discussion). PI3K can be activated by multiple signalling pathways. In ES cells FGF4 produced by the cells themselves (see section 3.2.3) could activate PI3K

through FGF receptors. To test the contribution of this the potent FGFR inhibitor, PD173074, was used. Reduction of pERK confirmed that the FGFR was effectively inhibited but there was no effect on basal or insulin-stimulated pPKB levels (Fig3.3f). This suggests that FGFs do not play a significant role in the activation of PI3K and that ES cells possess FGF- and insulin-independent PI3K activity, perhaps through Eras (Takahashi et al., 2003). However, the upregulation of pPKB in response to PD03 is abolished by PD17 (Fig3.3f) demonstrating that FGF signalling is required for this response. PS had no discernible effect on pPKB in the presence or absence of insulin (Fig3.3f) in agreement with the effect of combining the alternative MEK- and FGFR-inhibitors PD03 and PD17. Note the difference in pPKB activity between PD03 and PS (ie. 2i and 3i) indicates that these two media compositions do not behave identically although phenotypically there is little difference.

GSK3 is inhibited by phosphorylation on residues serine 9 (GSK3β) and 21 (GSK3α) (Ser9/21) (Doble and Woodgett, 2003). These residues are phosphorylated by PKB (Cross et al., 1995) and p90RSK (Saito et al., 1994) placing GSK3 downstream of PI3K and MAPK signalling, respectively. Inhibition of MAPK signalling by PS or PD03 may therefore have affected GSK3 activity. However, although phospho-Ser9/21 was readily detected, levels of phosphorylation were largely unaffected in steady-state cultures in the presence of the inhibitors (Fig3.3g). Counter-intuitively, Ser9/21 phosphorylation appeared to be slightly elevated in the presence of PD03. This may reflect increased PKB activity as anticipated from the observed increase in PKB-Ser473 phosphorylation in the presence of PD03 (Fig3.3f). CHIR appeared to reduce the total amount of GSK3 present in the cells while LIF had little discernible effect on total GSK3 or on phosphorylation levels (Fig3.3g). The basal level of GSK3 phosphorylation is likely attributable to active PI3K signalling, driven by insulin present in N2B27.

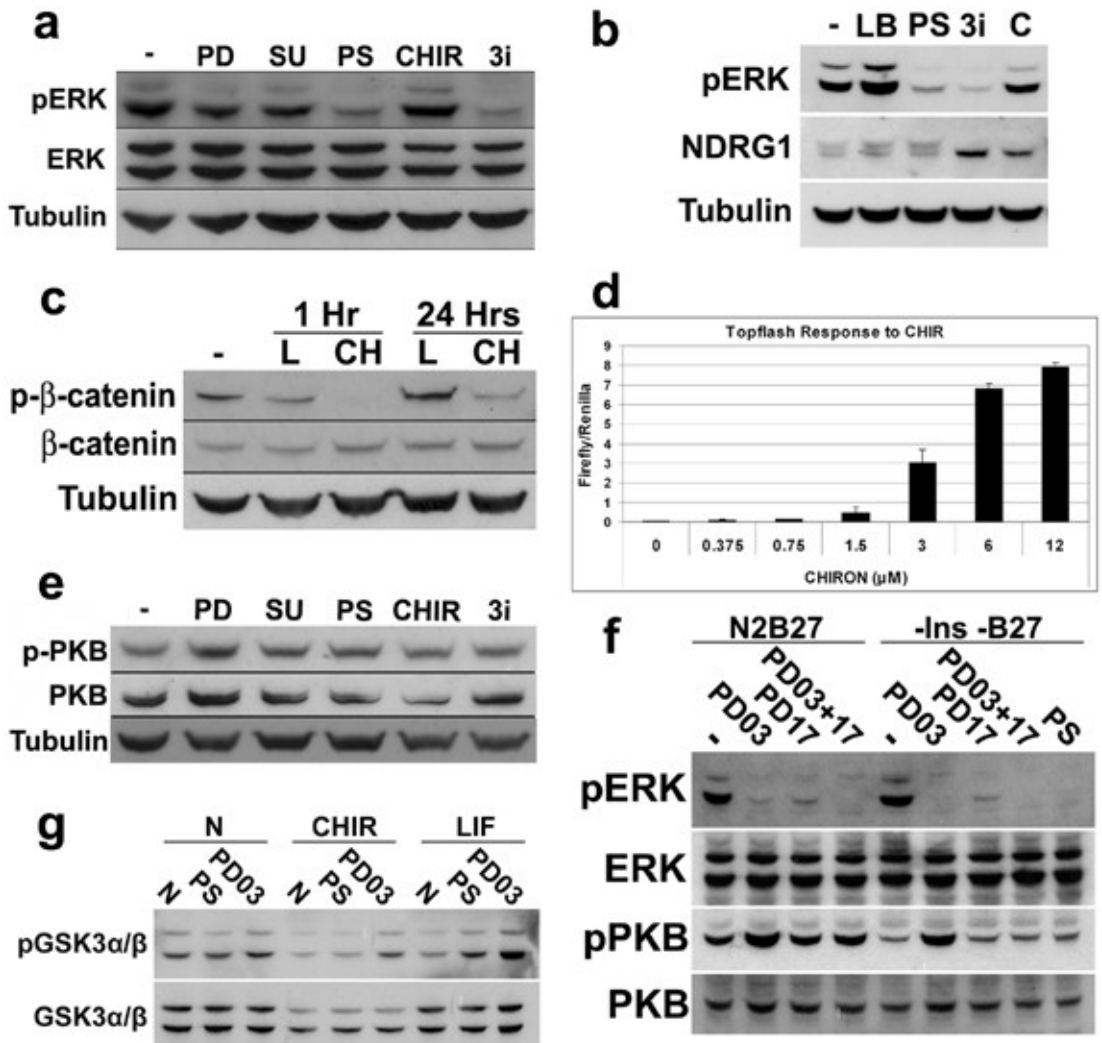


Fig3.3. Signalling pathway activation status in 3i. a) Immunoblot showing levels of phospho(Thr202, Tyr204)-ERK (pERK) in E14Tg2a ES cells cultured for 24hrs in N2B27 alone (-) or plus PD184352, 0.8μM (PD); SU5402, 2μM (SU); PD184352, 0.8μM plus SU5402, 2μM (PS); CHIRON99021, 3μM (CHIR) or 3i. Total ERK and Tubulin antibodies were used to control for loading. b) Immunoblot showing levels of pERK and NDRG1 in E14Tg2a ES cells cultured for 24hrs in N2B27 alone or plus LIF and BMP4 (LB); PS; 3i or CHIRON99021, 3μM (C). Tubulin was used to control for loading. c) Immunoblot showing levels of p-β-catenin and total β-catenin in E14Tg2a ES cells cultured for 24hrs in N2B27 alone or plus LIF (L) or CHIR99021, 3μM (CH) for 1 or 24hrs. Tubulin was used to control for loading. d) TOPFlash activity in E14Tg2a ES cells cultured overnight in N2B27 alone followed by 8hrs in N2B27 plus CHIR at the indicated concentrations. e) Immunoblot showing levels of phospho(Ser473)-PKB (p-PKB). Conditions as for (a). Total PKB and Tubulin antibodies were used to control for loading. f) Immunoblot showing levels of pERK and pPKB in E14Tg2a ES cells cultured for 24hrs in N2B27, or N2B27 without insulin or B27 (-Ins, -B27), alone or plus PD0325901, 1μM (PD03); PD173074, 0.1μM (PD17); both PD03 and PD17 or PS. CHIR present in all conditions. Total ERK and PKB antibodies were used to control for loading. g) f) Immunoblot showing levels of pERK and phospho(Ser9/21)-GSK3 in E14Tg2a ES cells cultured for 24hrs in N2B27 alone (N) or plus PS or PD03 in the presence of CHIR or LIF. Total ERK, total GSK3 and tubulin antibodies were used to control for loading.

3.2.3 The effect of SU5402 and PD184352 is mediated through inhibition of the FGF4-MEK-ERK signalling pathway

As discussed above, a major concern in the use of pharmacological inhibitors is off-target effects (Bain et al., 2007). To have confidence in the interpretation of experiments employing these inhibitors researchers should test alternative, structurally distinct inhibitors of their pathway in expectation that the results will be the same and should demonstrate that the effect of the inhibitor is dependent on the presence of the molecular target by complementary genetic studies. To this end alternative inhibitors have been used for each of the three included in 3i and the phenotype of ES cells either lacking the molecular target or with decreased levels of activity in the pathway in question examined.

The FGFR inhibitor SU is included in 3i at a concentration of 2μM. I examined whether an alternative inhibitor of the FGFR, PD17, could substitute for SU. I found that PD17 could substitute for SU at significantly lower concentrations, consistent with its higher potency. Furthermore, PD17 appeared sufficient to replace PS, supporting self-renewal in combination with CHIR (Fig3.4a), even at the single cell level (Fig3.2c). The sufficiency of PD17 likely reflects its potent inhibition of the FGFR and the significant down-regulation of pERK observed even at relatively low concentrations (Fig3.4b). However, colonies formed in the presence of PD17 tended to be smaller, perhaps indicating that PD17 affects the activity of pathways that promote ES cell growth or exhibits a degree of non-specific toxicity. An obvious candidate is PI3K which is activated downstream of the FGFR (see discussion).

ERK1/2 signalling can be activated by a range of cytokines and growth factors including LIF (Burdon et al., 1999b) and FGFs (reviewed in (Eswarakumar et al., 2005)). The effect of FGFR inhibitors on ERK activation in defined conditions strongly suggests that FGF ligands are largely responsible for activation of this pathway. FGF4 is expressed by ES cells (Rathjen et al., 1990; Ma et al., 1992) under the control of the pluripotency-associated TFs Oct4 and Sox2 (Yuan et al., 1995) and I sought to examine whether this is responsible for the high background levels of pERK observed in ES cells. ES cells lacking *Fgf4* have been generated (Wilder et al.,

1997) and I assessed their molecular and phenotypic properties in defined growth conditions. Levels of pERK were significantly lower in *Fgf4*-null than in heterozygote ES cells and the activation of pERK by the addition of LIF indicated that these cells were not unable to activate ERK and that the reduction in pERK could be attributed to the absence of FGF4 (Fig3.4c). The reduced ERK activity predicts that the requirement for SU and PD18 should be reduced and indeed *Fgf4*-null ES cells could readily be propagated in CHIR alone while heterozygote controls underwent differentiation (Fig3.4d) and could not be maintained long-term in CHIR alone. No clear difference was discernible between null and heterozygote lines grown in LIF and BMP4 or in 3i indicating that the heterozygote cells were not inherently compromised in their ability to self-renew. These findings were supported by the observation that *Fgf4*-null cells formed colonies from single cells in CHIR alone with much higher efficiency than heterozygote controls (Fig3.4e).

Further support for the specificity of SU and PD18 for the FGF-MEK-ERK signalling cascade came from the use of a more potent inhibitor of MEK, PD03 (Bain et al., 2007). PD03 blocked the activation of ERK similarly to PS when used alone at a concentration of around 1μM (Fig3.4f). Accordingly, the addition of 1μM PD03 and 3μM CHIR to N2B27 in the absence of FGFR inhibitors supported self-renewal of ES cells with a similar efficiency to 3i as judged by single-cell colony formation (Fig3.2c,d) and long-term bulk culture (Fig3.4g). This combination of inhibitors is termed ‘2i’. The removal of the requirement for FGFR inhibition demonstrates that inhibition of other signalling pathways activated by the FGFR is not critical for self-renewal in 3i and that the key downstream targets are likely to be ERK1/2.

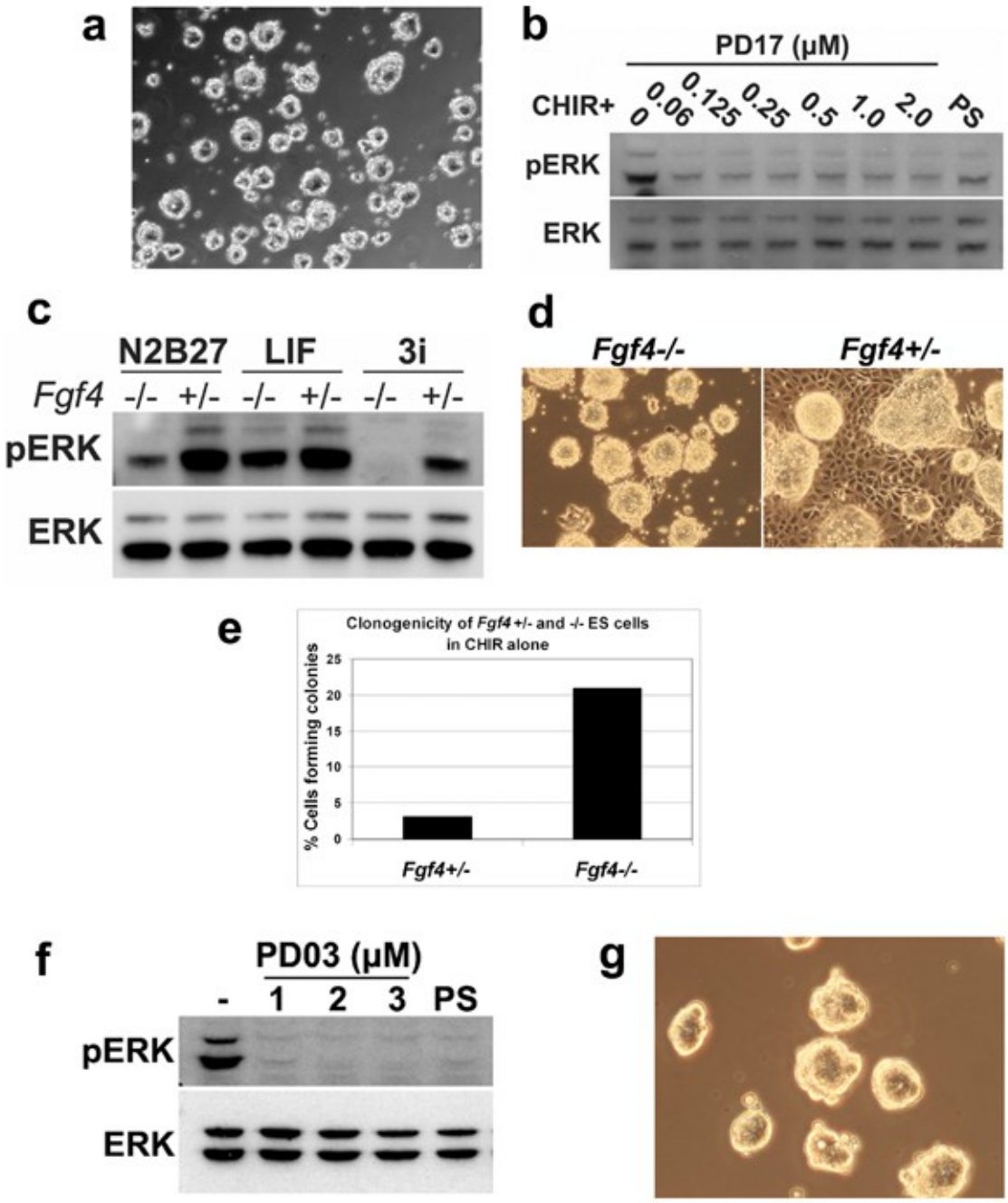


Fig3.4. PD184352 and SU5402 specifically inhibit the FGF4-MEK-ERK signalling pathway. a) Phase contrast image of E14Tg2a ES cells cultured in N2B27 plus CHIRON99021, 3μM and PD173074, 0.125μM. b) Immunoblot showing pERK levels in E14Tg2a ES cells cultured in N2B27 plus CHIR, 3μM and PD17 at the indicated concentrations or PS. Total ERK was used to control for loading. c) Immunoblot showing pERK levels in *Fgf4* homozygous null (-/-) or heterozygous (+/-) ES cells cultured for 24hrs in N2B27 alone or plus LIF or 3i. Total ERK was used to control for loading. d) Phase contrast pictures of *Fgf4* -/- or +/- ES cells after 4 passages in N2B27 plus CHIR (3μM) alone. e) Histogram showing percentage of single *Fgf4* -/- or +/- ES cells forming ES colonies in N2B27 plus CHIR (3μM) alone. f) Immunoblot showing pERK levels in E14Tg2a ES cells cultured in N2B27 alone or plus PD03 at the indicated concentrations or PS. g) Phase contrast image of E14Tg2a ES cells cultured in N2B27 plus CHIR, 3μM and PD03, 1μM (2i) for 4 passages.

3.2.4 The inhibitor CHIR99021 specifically targets GSK3

To inhibit GSK3 CHIR99021, the best characterised and most specific of the available GSK3 inhibitors (Murray et al., 2004; Bain et al., 2007), was used. As a control for the specificity of the phenotype for GSK3 two alternative GSK3 inhibitors, SB21 and SB41 (Smith et al., 2001), were tested for their ability to promote self-renewal in combination with PS. ES cells were cultured in PS plus SB21 or SB41 at a range of concentrations and assessed by morphology and growth rate to establish the most effective concentration. SB21 was found to support self-renewal at a concentration of approximately 4μM, the ES cells acquiring a similar morphology to those grown in 3i and retaining expression of pluripotency markers (Fig3.5a)**.** At higher concentrations of SB21 the colonies formed were smaller and the cells proliferated poorly upon passaging. In contrast, SB41 had no discernible effect on cellular phenotype. Both compounds were tested for their ability to activate the TCF/LEF reporter. SB41 failed to activate the reporter even at higher concentrations (Fig3.5b). It is not clear why it had no effect but these results explain the lack of a cellular phenotype. SB21 produced robust activation of the TCF/LEF reporter in a dose-dependent manner (Fig3.5b). Notably, the concentration optimised for cell culture activates TCF/LEF to a similar degree to 3μM CHIR suggesting that incomplete GSK3 inhibition is optimal for self-renewal.

ES cells have been engineered that have both alleles of both isoforms of GSK3 targeted and do not therefore express any functional GSK3 – they are referred to as double knockout (DKO) cells (Doble et al., 2007). Their study revealed considerable redundancy between the two GSK3 isoforms with a clear phenotype becoming apparent only when 3 of the 4 alleles had been inactivated. These cells provide an excellent control for the effect of CHIR. However, the lack of GSK3 confers a strong phenotype on the cells. They have highly elevated levels of β-catenin (Doble et al., 2007) (Fig3.5c) and correspondingly high tonic expression of β-catenin/TCF target genes *Axin2, Brachyury* and *Cdx1*, and highly elevated TCF/LEF reporter activity (Doble et al., 2007) (Fig3.5d). The cells have a highly refractile, tightly packed colony morphology and although it is not mentioned by the authors they have a high

degree of spontaneous differentiation with differentiated cells surrounding the undifferentiated colonies (Fig3.5e). Paradoxically, the cells resist differentiation in EBs with *Oct4* and *Nanog* expression persisting long after they have been lost from wild-type controls (Doble et al., 2007). In accordance with this DKO cells could be passaged several times in the absence of cytokines (Fig3.5e) but could not be maintained indefinitely. In a range of other conditions tested including the standard LIF and BMP4 the cells could be cultured long-term but spontaneous differentiation was always high (Fig3.5e; 3.6.1a,b). It was predicted that in the absence of GSK3 the requirement for CHIR would be lost in 3i or 2i culture and that the cells would self renew in PS or PD03 alone. Indeed, it was found that spontaneous differentiation was eliminated by inhibition of the MEK-ERK signal (Fig3.5f; 3.6.1a,b) and that long-term cultures could be maintained. The emergence of differentiated cells from the refractile colonies, a high proportion of which were found to be Gata4 positive (Fig3.6a,b), was not evident in the presence of PS or PD03 (Fig3.6a,b). However, DKO cells grew more slowly under these conditions than wild-type cells cultured in 3i and appeared to be more readily maintained when cell density was kept relatively high. This suggests that the cells were in some way compromised by the absence of GSK3. This may be attributable to the elevated levels of β-catenin and associated high rates of transcription of differentiation-associated genes. To stringently test the capacity of these cells for self-renewal clonal density colony forming assays were performed. DKO cells formed undifferentiated colonies in N2B27 alone or in PS (Fig3.5g). This clearly demonstrates the self-renewal phenotype resulting from loss of GSK3 since wild-type ES cells undergo differentiation in N2B27 alone and cannot be cloned efficiently in PS alone (see chapter 4). As expected, PS increased the proportion of totally undifferentiated colonies, indicating that differentiation was inhibited. Interestingly, self-renewal was enhanced by the addition of LIF to PS (Fig3.5g) or PD03 (Fig3.6b) demonstrating that LIF can activate signalling pathways that promote self-renewal independently of GSK3 and that DKO cells are not intrinsically compromised by the absence of GSK3. Addition of CHIR to these cells had no discernible effect on the cellular phenotype or on TCF/LEF reporter activity (Fig3.5d) and did not enhance colony forming efficiency (Fig3.5g) indicating that its effect on wild-type cells was dependent on the presence of GSK3.

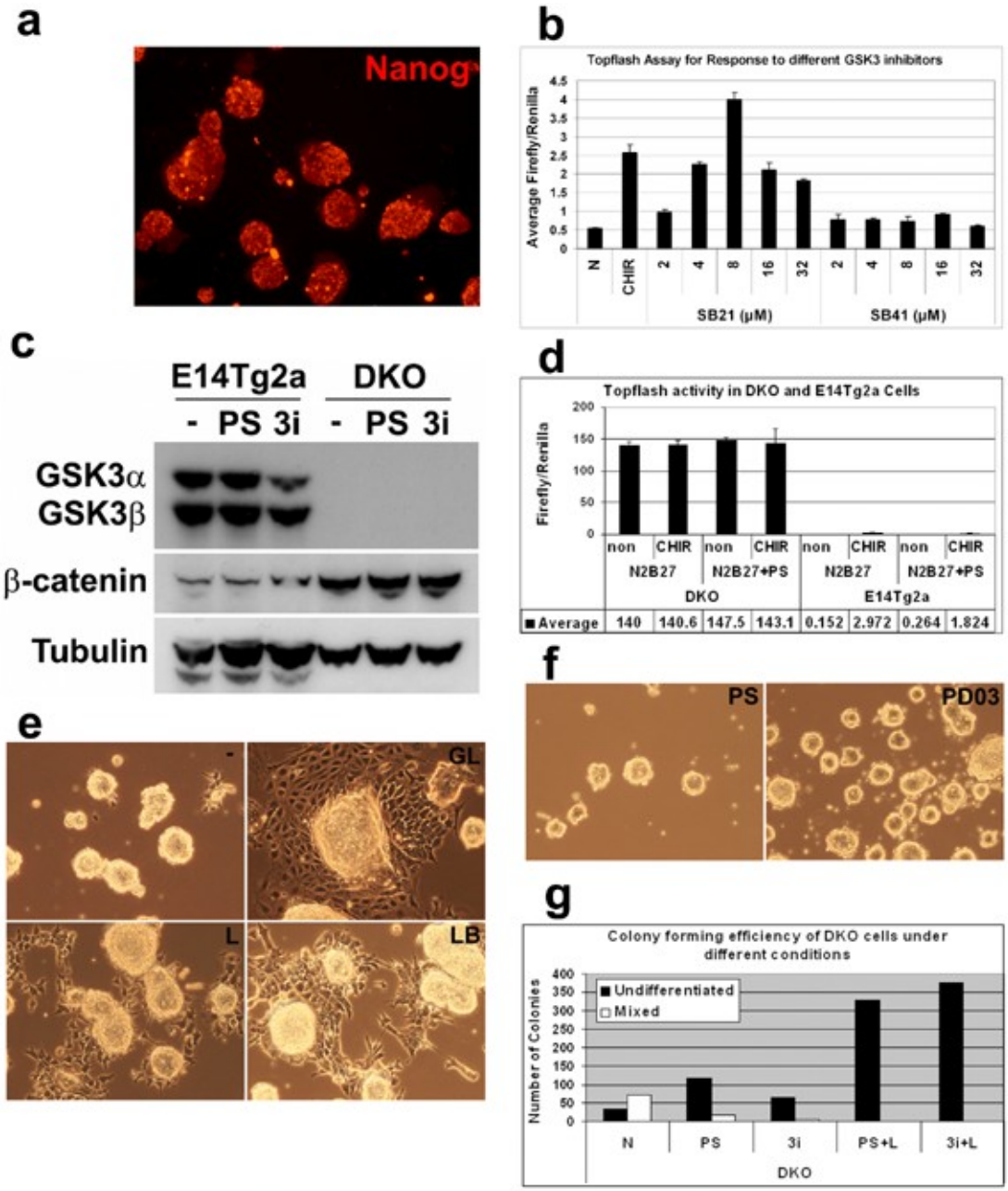


Fig3.5. CHIRON99021 specifically inhibits GSK3. a) Immunostaining showing Nanog expression in E14Tg2a ES cells grown for 4 passages in PS plus SB216762, 4μM (SB21). b) Histogram showing TOPFlash activity in E14Tg2a ES cells cultured overnight in N2B27 alone (N) and a further 8hrs in N2B27 plus CHIRON99021, 3μM (CHIR); SB216762, (SB21) or SB415286 (SB41) at the indicated concentrations. c) Immunoblot showing levels of GSK3 and β-catenin in E14Tg2a or DKO ES cells cultured for 24hrs in N2B27 alone (-) or plus PS or 3i. Tubulin was used to control for loading. Note background bands on Tubulin blot result from incomplete stripping of the GSK3 antibody. d) Histogram showing TOPFlash activity in GSK3 double knockout (DKO) and wild-type E14Tg2a ES cells cultured overnight in N2B27 alone and a further 8hrs in N2B27 alone (non) or plus CHIRON99021, 3μM (CHIR) in the presence or absence of PS. Note that neither CHIR nor PS influence TOPFlash activity in DKO cells. e) Images of DKO cells after 4 passages in N2B27 alone (-), serum plus LIF (GL), LIF alone (L) or LIF and BMP4 (LB). f) Images of DKO cells after 8 passages in N2B27 plus PS or PD0325901, 1μM (PD03). g) Histogram showing number of undifferentiated (alkaline phosphatase (AP) positive) and mixed colonies formed from 600 DKO ES cells plated at clonal density in N2B27 alone (N) or plus PS or 3i in the presence or absence of LIF (L).

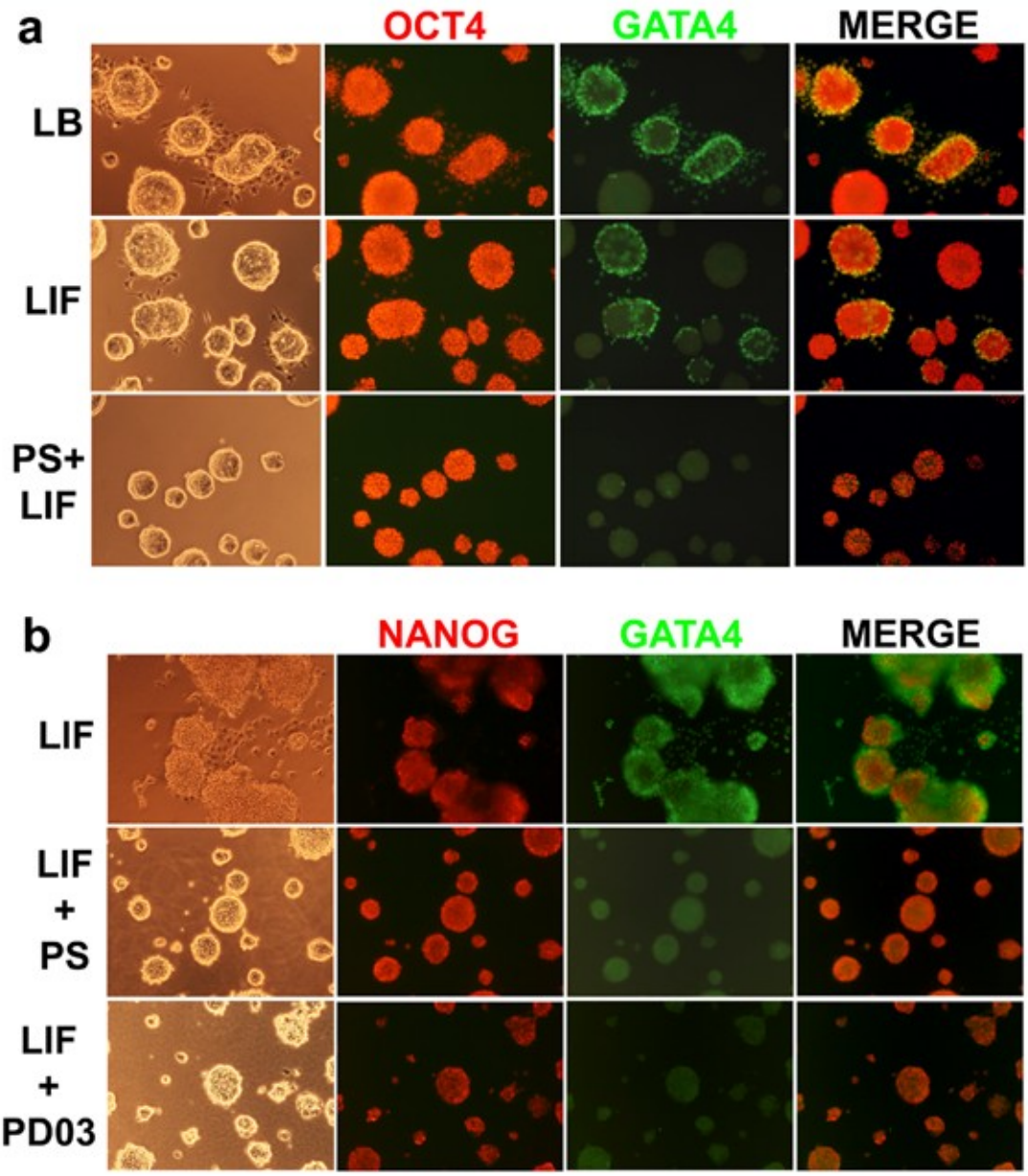


Fig3.6. ERK inhibition but not LIF blocks differentiation in DKO cells. a) After 2 passages in N2B27 plus LIF and BMP4 (LB); LIF or LIF plus PS GSK3 double knockout (DKO) cells were fixed and stained for Oct4 and Gata4. Note almost complete absence of Gata4 positive cells in the presence of PS. b) After 6 passages in N2B27 plus LIF; LIF plus PS or LIF plus PD03 DKO cells were fixed and stained for Gata4 and Nanog. Note complete absence of Gata4 positive cells in the presence of PS or PD03.

3.2.5 Embryonic Stem Cell Self-Renewal in 3i is LIF and BMP4 Independent

To date two signalling pathways have been clearly demonstrated to be capable of supporting self-renewal: LIF-STAT3 (Niwa et al., 1998; Burdon et al., 1999b; Matsuda et al., 1999) and BMP-Id (Ying et al., 2003a). I investigated whether signalling through these pathways is required for self-renewal in 3i.

BMP4 is added to serum-free ES cell cultures to prevent neural commitment. It has been demonstrated that induction of *Id* genes through BMP-Smad1/5 signalling is sufficient to mediate this effect (Ying et al., 2003a) but it has also been suggested that BMP signalling results in a reduction in ERK and p38 activity and that this is responsible for the pro-self-renewal effect of BMP4 (Qi et al., 2004). Both possibilities were investigated in the context of 3i. Transcript levels of *Id1* were significantly lower in 3i than in LIF and BMP4 (Fig3.7a). It has previously been shown that PD18 or SU can replace the requirement for BMP4 (Ying et al, in press). The effect of PS on Id expression was therefore assessed. Reduced phospho-ERK clearly shows that the inhibitors are active but there was no evidence of any effect on Id expression (Fig3.7b). Similarly, Id levels were clearly increased in the presence of BMP4 but BMP4 had no effect on the steady state levels of ERK or p38 phosphorylation compared to N2B27 or LIF alone (Fig3.7b). Therefore these pathways are unlikely to be effectors downstream of BMP signalling, consistent with previous conclusions (Ying et al., 2003a).

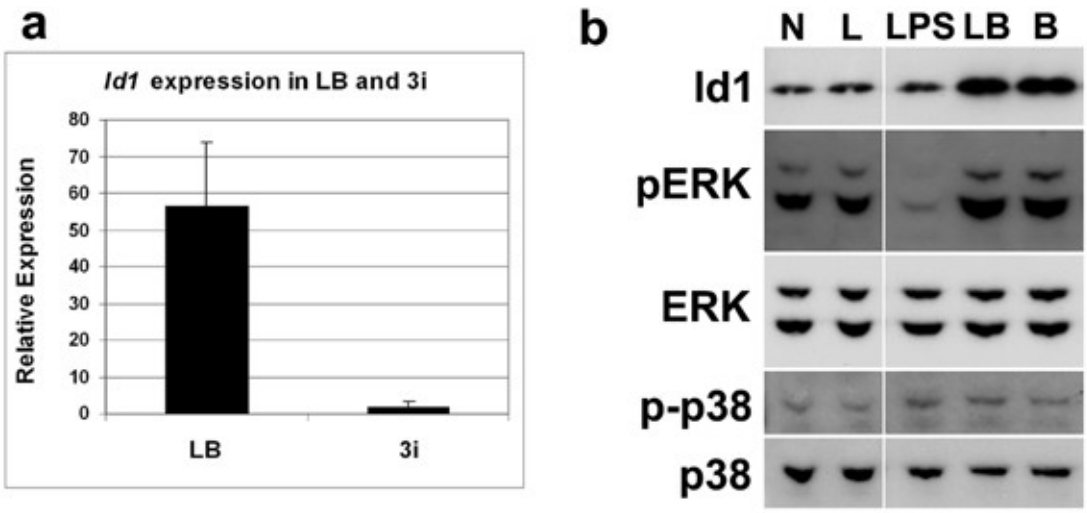


Fig3.7. Elevated BMP-Id signalling is not required for ES cell propagation in 3i. a) Histogram showing relative levels of *Id1* expression in ES cells cultured for 24hrs in LB or 3i. b) Immunoblot showing levels of Id1, pERK and p-p38 in E14Tg2a ES cells cultured for 24hrs in N2B27 alone or plus LIF (L); LIF and PS, LPS; LB or BMP4 (B). Total ERK and p38 antibodies were used to control for loading. 2 lanes have been deleted to exclude a technical error but samples shown were run on the same gel

Prior to commencing this project it had already been shown that ES cells grown in 3i do not have detectable levels of phosphorylation on Tyr705 of STAT3 (Ying et al, submitted). Phosphorylation at this site is considered essential for canonical activation of STAT3 (reviewed in (Darnell et al., 1994)) but it has been reported that STAT3 phosphorylation at Ser727 can promote self-renewal in mouse NS cells and hES cells (Androutsellis-Theotokis et al., 2006). I therefore undertook to further investigate the requirement for LIF-Stat3 in 3i by genetic methods. Cells lacking the LIFR (Hyg34) are normally maintained in the presence of IL6 and its soluble receptor which engage the gp130 receptor and activate STAT3 (Yoshida et al., 1996; Dani et al., 1998). These cells differentiate in LIF but are readily maintained in 3i. RT-PCR analysis of marker expression in ES cells maintained in LB, 3i or IL6 shows that pluripotency markers are down-regulated in LB, as expected, while the differentiation marker *Brachyury* is upregulated (Fig3.8a). In contrast, cells maintained in 3i or IL6 remained undifferentiated. This demonstrates that cells grown in 3i do not depend on paracrine LIF signalling. *Socs3* is a well characterised transcriptional target of STAT3 (Auernhammer et al., 1999). Real-time PCR for

steady state levels of the *Socs3* transcript revealed that they were significantly lower in 3i than in LIF and BMP4 (Fig3.8b), indicating reduced activity of STAT3.

As there remains the possibility that STAT3 has functions separate from its role as a TF we attempted to derive ES cells carrying a deletion of the *Stat3* gene. Mice heterozygous for *Stat3* were crossed, ICMs isolated from expanded blastocysts and plated on a feeder layer of MEFs in 3i (derivation and initial cell culture by J. Nichols, Ying et al, in press). Several lines were isolated and PCR genotyped revealing several heterozygous and one homozygous null line (Fig3.8c). One heterozygous and the homozygous null line were used for further analysis. A wild-type line established independently from the same mouse strain (J. Nichols) served as a further control. To confirm the absence of STAT3 from these cell lines immunoblotting was performed. STAT3 was undetectable in the null line and the heterozygous line expressed low levels of STAT3 compared to the wild-type control. Similar levels of Oct4 in all of the lines (Fig3.8d) indicated that they remained undifferentiated. Morphologically, the lines were indistinguishable when maintained in 3i and all exhibited reduced substrate attachment necessitating their growth on feeders or on laminin. The fact that they can be maintained on laminin demonstrates that the feeder layer is not strictly required to maintain these ES cells and that 3i culture conditions are sufficient. *Stat3* heterozygous and null cells grown in 3i retained expression of pluripotency markers as judged by immunostaining for Oct4 (Fig3.8e) and RT-PCR analysis of pluripotency markers (Fig3.8f).

As further indication of the loss of STAT3 activity transcriptional responses to LIF were examined by real-time PCR. While heterozygous cells upregulated both *Socs3* (STAT3 target) and *Egr1* (ERK target (Weinhold et al., 2000)) in response to LIF, null cells upregulated only *Egr1* (Fig3.8g) indicating that the ERK signalling arm was left intact while STAT3 signalling was abolished. When transferred to standard ES cell growth conditions (serum plus LIF on MEFs) the wild-type line efficiently formed undifferentiated colonies while both the heterozygous and null lines underwent rapid differentiation and failed to give rise to any morphologically undifferentiated colonies (Fig3.8h). This indicates that Stat3 exhibits

haploinsufficiency, presumably because the lower levels of Stat3 observed in these cells (Fig3.8d) are not sufficient to balance the differentiation-inducing MEK-ERK signal. Low STAT3 levels in the heterozygous cells may be confounded by maintenance in 3i where expression levels of STAT3 were observed to be lower in wild-type cells when compared to LIF-containing culture conditions (Fig3.8d). *Stat3* is a transcriptional target of STAT3 itself so transcription is likely to be lower in 3i where STAT3 is inactive (Fig3.8b) (Ying et al, in press).

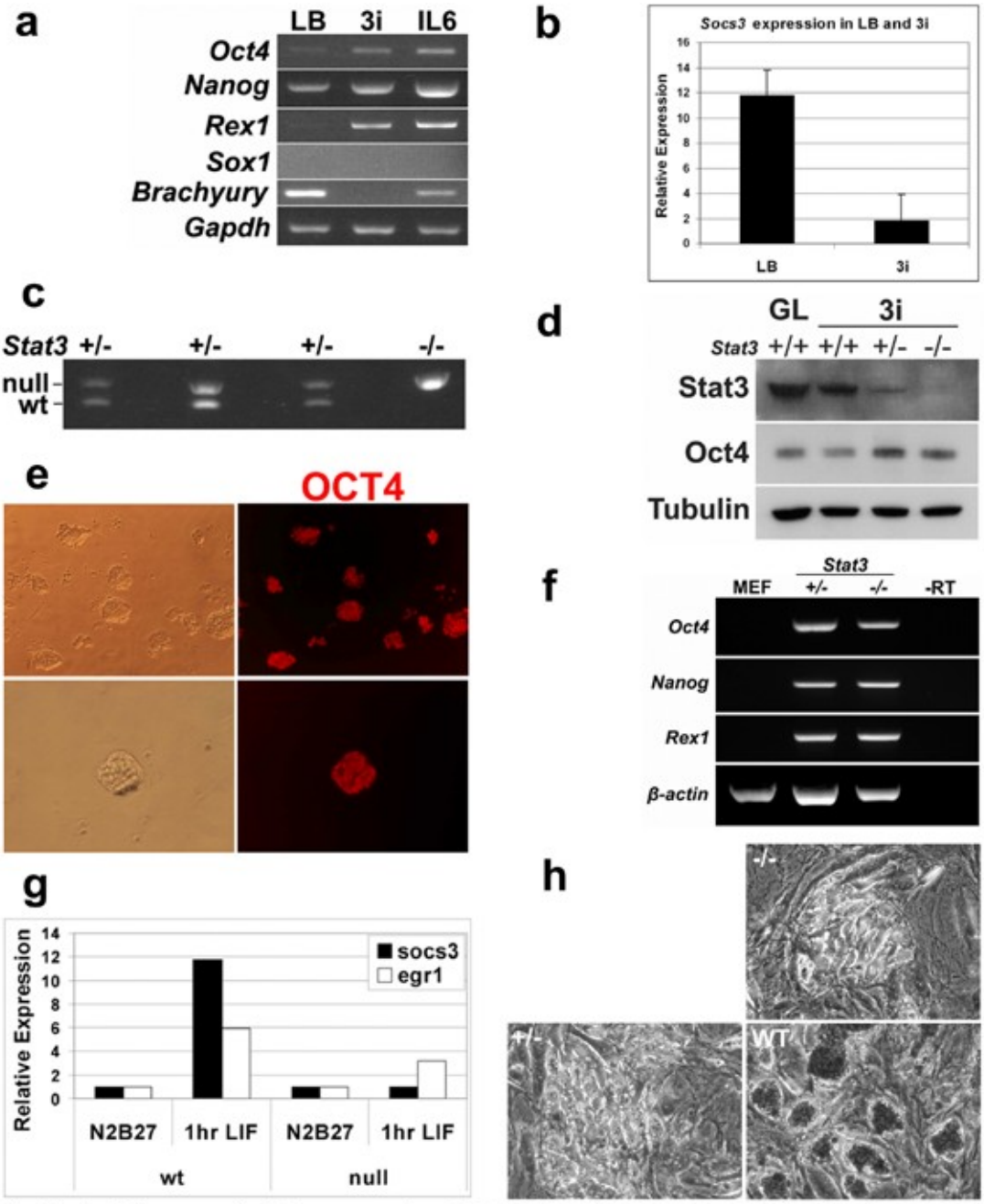


Fig3.8. Stat3 is not required for propagation of ES cells in 3i. a) RT-PCR analysis of marker expression in *LIFR*-/- (Hyg34) ES cells cultured in LIF and BMP4 (LB), 3i or IL6 plus sIL6R (IL6). RNA provided by Q-L. Ying. b) Histogram showing relative levels of expression of *Socs3* in E14Tg2a ES cells cultured in LB or 3i. Error bars represent standard deviation of the mean for 2 biological replicates. c) Genomic PCR genotyping of ES cells derived from Stat3+/- intercross embryos. Upper band indicates presence of the targeted (null) allele and lower band presence of the wild-type allele. d) Immunoblot showing STAT3 and Oct4 expression in *Stat3* +/+, +/- and -/- ES cells cultured in 10% serum plus LIF (GL) or 3i. Tubulin was used to control for loading. f) RT-PCR analysis of marker expression in mouse embryonic fibroblasts (MEFs) and *Stat3* +/- and -/- ES cells cultured in 3i. -RT denotes no reverse transcriptase control. g) Histogram showing relative expression levels of *Socs3* and *Egr1* in wild-type (wt) and *Stat3* -/- (null) ES cells cultured for 4hrs in N2B27 in the absence of cytokine followed by 1hr in N2B27 with or without LIF. h) Phase contrast images of leishmann-stained cultures of *Stat3* -/-, +/- or +/+ (WT) ES cells cultured on MEFs in GL for 1 passage. Note dark staining colonies of ES cells detected in WT cultures. In -/- and +/- cultures only differentiated colonies were observed which were difficult to distinguish from the feeder layer.

As a test of pluripotency *in vitro* differentiation of *Stat3*-null ES cells was examined. In suspension, in the presence of serum EBs were allowed to form and were harvested at days 3 and 6 for RNA isolation. Real-time PCR indicated up-regulation of the lineage associated genes *Brachyury* (*T*, marker of early mesoderm progenitors), and *Gata-4* and *6* (markers of endoderm) as the pluripotency associated genes, *Oct4, Nanog* and *Rex1* were down-regulated (Fig3.8.1a). Formation of EBs in serum-free conditions for approximately 10 days and plating onto laminin resulted in neuronal differentiation as indicated by immunostaining for the neuronal marker β-III Tubulin (Tuj1) and the clear neuronal morphology (Fig3.8.1b). Plating on gelatine in the presence of EGF and FGF2 after 10 days of serum-free suspension culture allowed derivation of neural stem (NS) cells (Conti et al., 2005) from *Stat3*-null ES cells. Genotyping by PCR (Fig3.8.1c) and immunoblotting (Fig3.8.1d) confirmed that the NS cells did not express STAT3. The NS cells were assessed for expression of a panel of immunohistochemical markers whose expression are associated with NS cell phenotype (Conti et al., 2005) (Fig3.8.1e,f). The cells were positive for Nestin, Blbp, Rc2 and Sox2, had morphology typical of adherent NS cells and proliferated rapidly. *Stat3*-null NS cells could provide a tool to examine the role of STAT3 in NS cells themselves and in differentiation into neurons, astrocytes and oligodendrocytes.

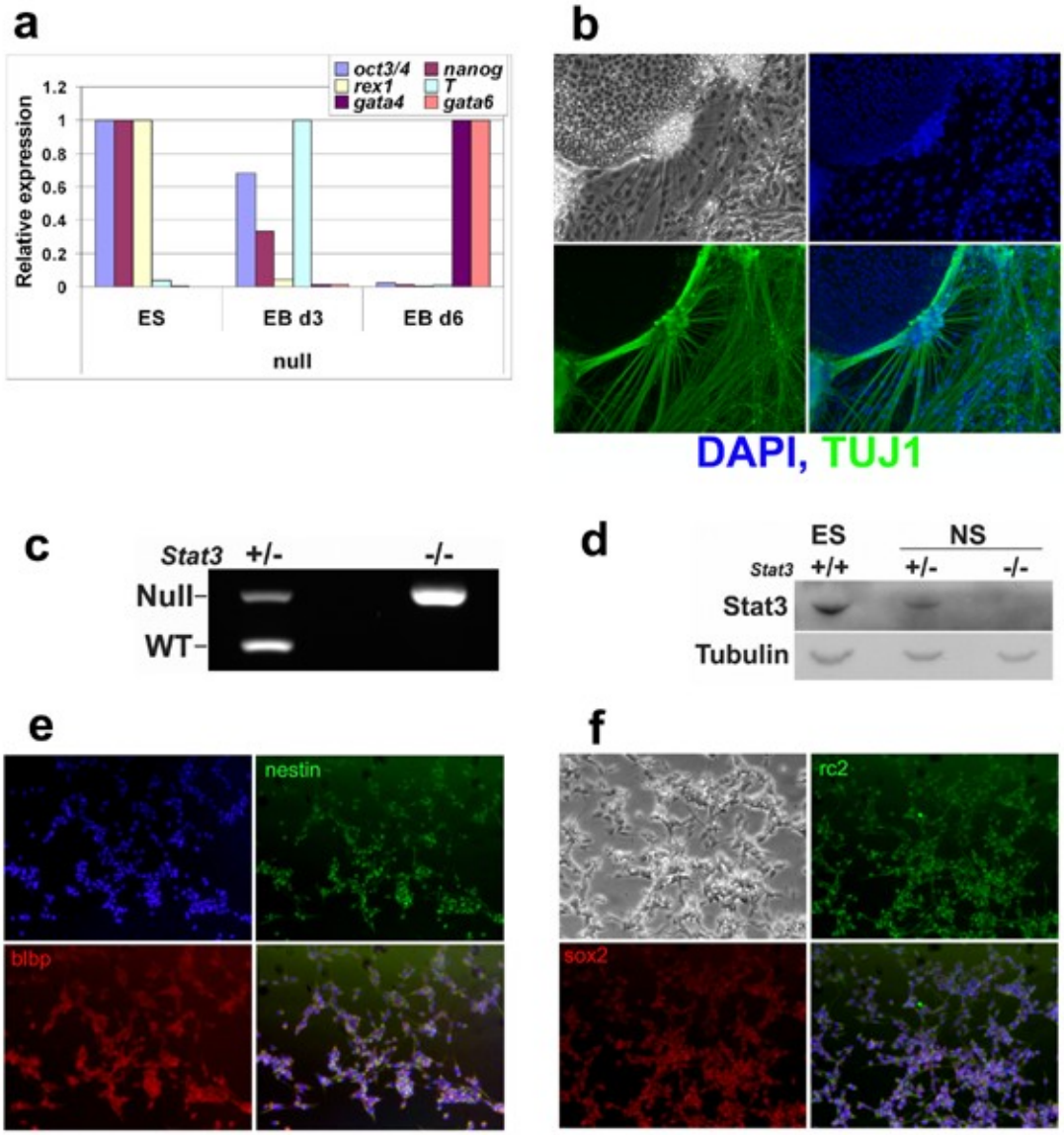


Fig3.8.1. *Stat3*-/- ES cells are pluripotent. a) Histogram showing real-time PCR analysis of marker expression in *Stat3*-/- ES cells (ES) and after 3 (d3) or 6 (d6) days of differentiation as embryoid bodies (EB). b) Immunostaining for the neuronal marker β-III-Tubulin (Tuj1) following plating of EBs formed in serum-free conditions onto laminin. Note neuronal morphology. c) PCR genotyping of *Stat3*+/- and -/- neural stem (NS) cells. d) Immunoblot showing Stat3 expression in *Stat3*+/- and -/- NS cells. WT ES cells (ES) were used as a positive control for Stat3. Tubulin was used as a loading control. e,f) Immunostaining showing expression of the NS cell markers Nestin and Blbp (e) and Rc2 and Sox2 (f) in *Stat3*-/- NS cells.

3.2.6 Are N2 and B27 Supplements Absolutely Required for Self-Renewal?

As a base for our serum-free culture systems the media N2B27 is used. To a combination of commercial medias, Neurobasal and Dulbecco's Modified Eagle

Medium (DMEM): F12, the commercial supplement B27 and an in house- prepared N2 (see materials and methods for composition) are added. N2 contains hormonal components capable of activating signalling pathways in the cell and while there is little evidence in the literature to suggest that these widely used supplements influence anything other than the growth and survival of the cells it is quite possible that in certain contexts the signalling pathways they activate become instructive in cell fate decisions. N2 contains high levels of insulin which activates PI3K signalling (Fig3.3e,f) through binding to its cognate receptor (reviewed in (Hawkins et al., 2006)) resulting in activation of downstream pathways known to be involved in cell cycle and proliferation and implicated in ES cell self-renewal (Takahashi et al., 2005). Previous work shows that despite significantly decreased viability ES cells can be maintained in a basal media, supplemented with BSA and transferrin, that does not contain insulin, for at least one month (Ying et al, in press) although under these conditions the cells show increased sensitivity to the inhibitors and reduced concentrations of PD18 (0.5μM) and SU (1μM) were used. This demonstrates that insulin is not strictly required for self-renewal.

B27 contains a range of defined components including free radical scavengers and anti-oxidants. I found that ES cells could be propagated long term in 3i when B27 was removed from the media (Fig3.9a) but that clonal propagation was not possible (Fig3.9c,d). As our cells are routinely grown in atmospheric oxygen they are likely to be subject to oxidative stress. Colony forming efficiency at oxygen concentrations closer to physiological levels (5%) was tested. Significantly, clonal propagation became possible in the absence of B27 (Fig3.9c,d) with two independent cell lines. Oct4GIP ES cells retained expression of their GFP transgene (Fig3.9b) indicating that the colonies formed remained undifferentiated. I conclude that B27 is not required for self-renewal but enhances viability, particularly when oxygen levels are high.

In the absence of B27 the media contains only the N2 supplement. N2 is fully defined with the exception of bovine serum albumin (BSA) fraction V. To eliminate the possibility that undefined components of BSA affect self-renewal BSA was

replaced with recombinant human serum albumin (rHSA) and the cloning efficiency of E14 IV C ES cells (a subclone of E14Tg2a recently tested for germ-line competency) assessed. As B27 was excluded from the media the experiment was performed under low O_2 conditions. Colonies with ES morphology were obtained using BSA or rHSA (Fig3.9f). Notably, clonogenicity was enhanced in media with rHSA as compared to BSA (Fig3.9e). This may reflect a degree of toxicity of undefined components in BSA. It was also possible to maintain long-term cultures in the defined media supplemented with PD03 and CHIR (2i). E14 IV C ES cells were cultured for 6 passages in a low O_2 incubator. Although proliferation was slow relative to cells cultured in full complement N2B27 there was little evidence of differentiation and immunostaining revealed that the cells retained expression of Oct4 and Nanog (Fig3.9.1a).

Colony forming efficiency was also examined in the absence of insulin (and B27), using reduced concentrations of PD03. Surprisingly, colonies formed with reasonable efficiency in the absence of insulin (Fig3.9e) indicating that insulin is not required for clonal ES cell growth. Once again, the efficiency of colony formation was slightly higher in media containing rHSA as compared to BSA. Colonies formed in the absence of B27 and/or insulin tended to be much smaller than those formed in full N2B27 media (Fig3.9f), probably reflecting a lower proliferation rate. Given that the clonal growth of ES cells in the absence of B27 and insulin is somewhat unexpected some of the colonies that had formed were passaged to assess their ability to expand. Upon passaging, colonies with ES cell morphology formed (Fig3.9.1b) but expanded extremely slowly and were not maintained beyond passage 3. The poor expansion of ES cells under these growth conditions appeared to result from a decrease in viability and proliferation rather than differentiation. The colonies remaining at passage 3 expressed Oct4 and Nanog as judged by immunostaining (Fig3.9.1c). Together, these data indicate that the supplements included in N2B27 serve to reduce oxidative stress and to enhance clonogenicity and cell proliferation but that they are not absolutely required for self-renewal.

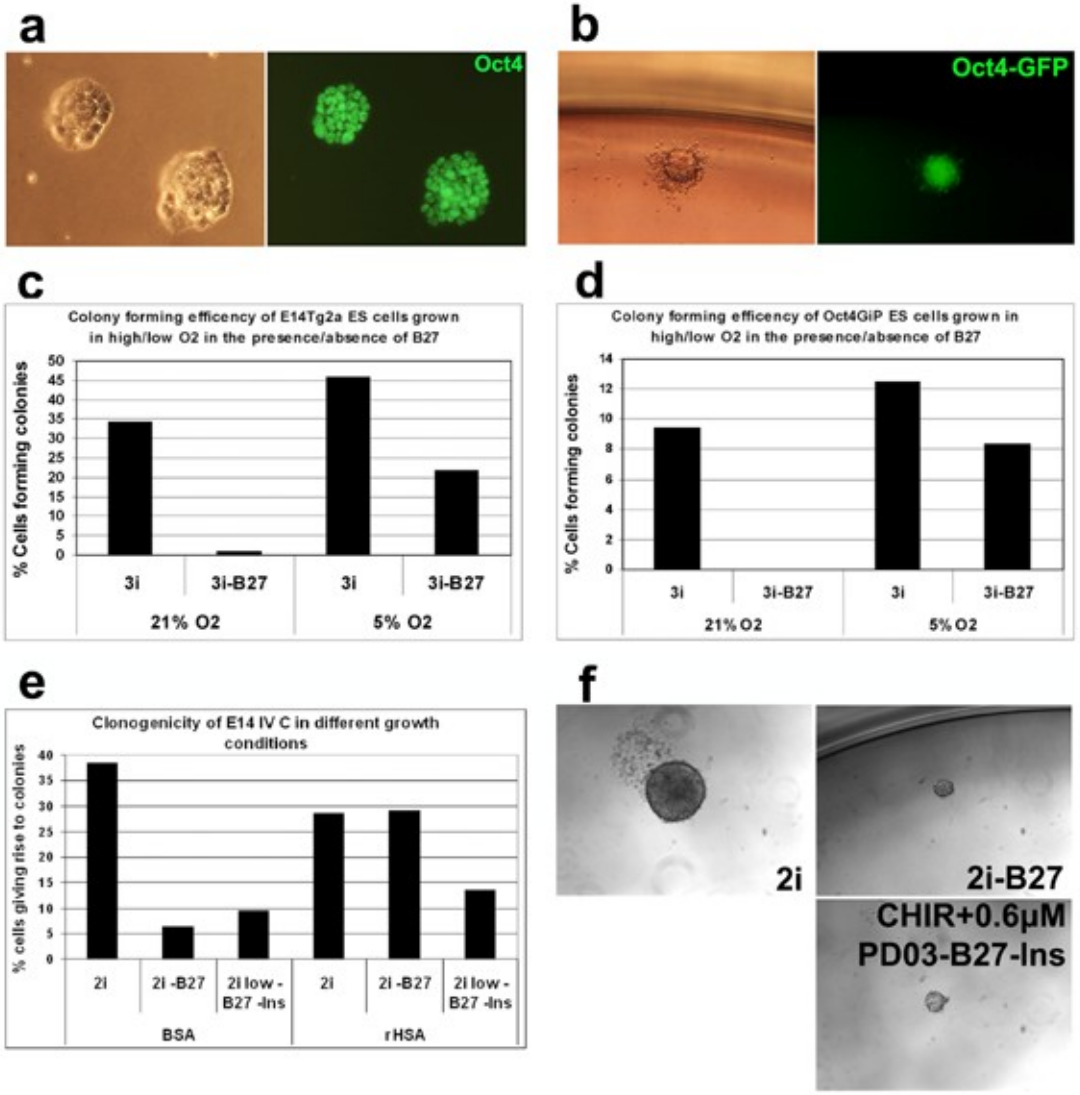


Fig3.9. B27 and insulin are dispensable for ES cell self-renewal in 3i. a) E14Tg2a ES cells were cultured for 6 passages in 3i in the absence of B27 and immunostained for Oct4. b) Colony formed from a single Oct4GIP ES cell in 3i in the absence of B27 at 5% oxygen. GFP is expressed from a transgene under the control of the *Oct4* promoter. c,d) Histograms showing percentage of E14Tg2a (c) and Oct4GIP (d) single cells forming ES colonies in 3i in the presence or absence of B27 at atmospheric or 5% oxygen (O2). e) Histogram showing percentage of E14 IV C single cells forming ES colonies in 2i in the presence or absence (-B27) of B27 and in 3µM CHIR plus 0.6µM PD03 (2i low) in the absence of B27 and insulin (Ins). Where indicated BSA in N2 was substituted for recombinant human serum albumin (rHSA). f) Representative phase contrast images taken at the same magnification of colonies formed in the indicated conditions using rHSA in place of BSA.

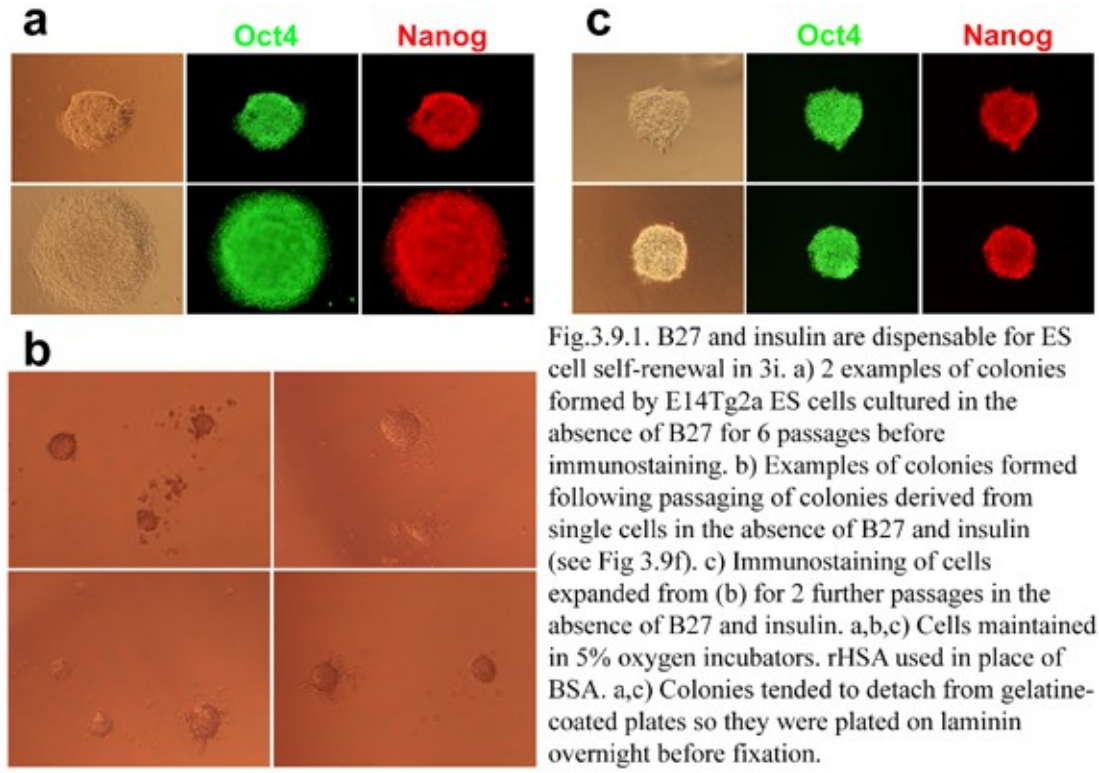


Fig.3.9.1. B27 and insulin are dispensable for ES cell self-renewal in 3i. a) 2 examples of colonies formed by E14Tg2a ES cells cultured in the absence of B27 for 6 passages before immunostaining. b) Examples of colonies formed following passaging of colonies derived from single cells in the absence of B27 and insulin (see Fig 3.9f). c) Immunostaining of cells expanded from (b) for 2 further passages in the absence of B27 and insulin. a,b,c) Cells maintained in 5% oxygen incubators. rHSA used in place of BSA. a,c) Colonies tended to detach from gelatine-coated plates so they were plated on laminin overnight before fixation.

3.3 Discussion

With proven ES cell culture conditions as a starting point I set out to characterise cells grown under these conditions with respect to signalling pathways previously implicated in ES cell self-renewal and to establish confidence in the mode of action of the three pharmacological inhibitors used in 3i. It is significant that the concentrations of inhibitors employed were optimised for ES cell culture before they were characterised by molecular biology. This approach means that there were no initial biases or assumptions directing the concentrations or combinations of inhibitors used. For example, CHIR is used at a dose that activates the TCF/LEF luciferase reporter sub-maximally but this became apparent only in retrospect.

3.3.1 3i supports robust self-renewal of ES cells

ES colonies in 3i assume a rounded morphology more typical of ES cells grown on feeders and often cited as a desirable quality in ES cell culture. When isolated single

cells are plated they form undifferentiated colonies with similar efficiency to cells plated in LIF and BMP4 in side-by-side experiments. Together with the previous demonstration that these conditions support derivation and culture of germ-line competent ES cells (Ying et al, in press) it is clear that 3i satisfies the criteria for sufficiency for self-renewal (Smith, 2001).

3.3.2 Signalling pathway activation in 3i

As an initial analysis of signalling pathway activity western blots were performed to assess activity in key proteins in these pathways. All three inhibitors affected their targets as predicted. Significantly, a combination of PD18 and SU (PS) appeared to mediate potent inhibition of ERK phosphorylation at concentrations that had relatively little effect independently. I surmise from this that to achieve the same phenotypic effect with either PD18 or SU alone it would be necessary to use significantly higher concentrations, increasing the chances of off-target and toxic effects on the cells. Toxicity has previously been noted by our group for the less potent MEK inhibitors PD98059 and U0126 and may explain why it has not previously been possible to establish growth conditions based on MEK inhibition. Thus PS may provide a sufficient level of ERK inhibition while minimising negative effects of the two inhibitors on ES cell culture. CHIR blocked phosphorylation of its well characterised targets, NDRG1 and β-catenin, and induced activation of the TCF/LEF reporter construct. Note that the concentration of CHIR used in cell culture (3μM) does not maximally activate the TCF/LEF reporter implying that GSK3 activity is not totally blocked. In support of this, cells lacking GSK3 (DKO cells) grew less well in MEK inhibitor or PS alone than wild-type cells cultured in 3i or 2i. This may be attributable to the elevated activity of canonical Wnt targets in these cells.

Phosphorylation of PKB was high in all insulin-containing media tested with no evidence that the inhibitors affected its activity with the exception of PD03 which caused increased phosphorylation of PKB. While it cannot be ruled out that PI3K activity is required for self-renewal from these experiments note that cells cultured in

the absence of inhibitors in N2B27 have high PI3K activity but undergo efficient neural commitment (Ying et al., 2003b) and that 3i mediates inhibition of differentiation without significantly altering levels of PKB activity. I have shown that PI3K signalling is activated by insulin in serum-free culture conditions. A non-essential role for insulin in self-renewal is supported by the observation that in its absence ES cells can be propagated in 3i for at least one month and can form clones from single cells albeit with reduced clonogenicity and growth rate. However, the removal of insulin from the media does not mean that the PI3K pathway becomes inactive. The data provided in this chapter indicate that MEK-inhibition results in increased PKB phosphorylation in the absence of insulin. ES cells have some constitutive activation of this pathway as a result of the activity of ES cell-specific Ras, Eras (Takahashi et al., 2003) and PI3K may also be activated by FGF4 through the FGFR (reviewed in (Eswarakumar et al., 2005)). The effect of FGFR inhibition proved to be informative. In insulin-containing or insulin-free media PD17 alone had no effect indicating that activation of PI3K through the FGFR was not significant in this context and that phosphorylation of PKB was likely to be FGF-independent, probably resulting from Eras activity (Takahashi et al., 2003). However, when PD17 was added together with PD03 it abolished the upregulation of pPKB. This shows that the effect of the MEK inhibitor is dependent on activation of the FGFR and probably reflects autoregulation of MAPK signalling. It has been shown that ERK can phosphorylate the docking protein FRS2α on threonine residues and that this negatively regulates the interaction with Grb2 and hence coupling to PI3K and MAPK pathways through Gab1 and Sos respectively (Lax et al., 2002) (Fig3.10). Thus, MEK inhibitors will decrease ERK activity and FRS2α phosphorylation, and increase activity immediately downstream of the FGFR. This suggests an explanation for the increase in PKB activity in response to PD03 but not PD17. While both inhibitors reduce ERK activity, PD17 directly inhibits the FGFR and hence its interaction with FRS2α, blocking activation of both PI3K and MAPK pathways.

Elevated PKB activity is likely to be responsible for the modest increase in GSK3 phosphorylation observed in the presence of PD03. Notably, however, GSK3 Ser9/21 phosphorylation is largely unaffected by other inhibitors or by LIF under these

conditions. This suggests that their effects are not mediated through regulation of GSK3 activity. However, the slight decrease in GSK3 activity anticipated in the presence of PD03 may in part explain small phenotypic differences between cells cultured in PD03 and PS (see chapter 4).

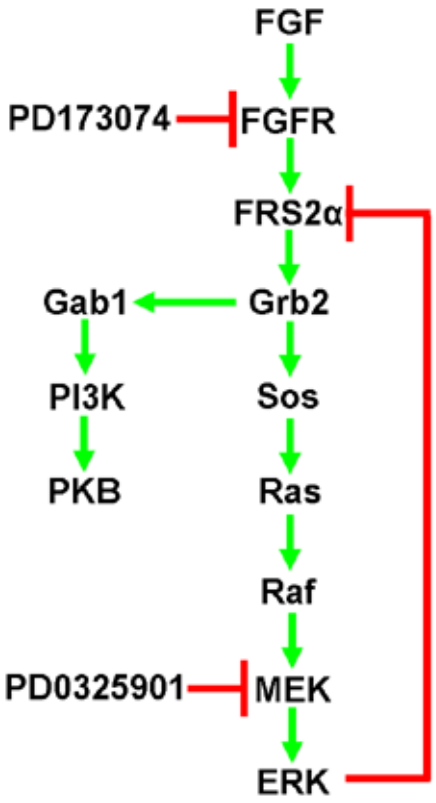


Fig3.10. FGF signalling, adapted from Eswarakumar et al, 2005. FGF activates the MAPK and PI3K pathways through FRS2α and Grb2. Phosphorylation of FRS2α by ERK inhibits recruitment of Grb2 and hence Sos and Gab1. PD0325901 inhibits MEK reducing ERK activity and relieving FRS2α from inhibition resulting in increased PI3K signalling. PD173074 directly inhibits the FGFR resulting in a reduction in all downstream signalling.

These observations pave the way for further investigation of ERK and PKB signalling in ES cells. I predict that the basal levels of pPKB will be abolished in *Eras*-/- ES cells (Takahashi et al., 2003) and that upregulation of pPKB in response to MEK inhibition will be reduced or abolished in *Fgf4*-/- ES cells. It should be possible to correlate the activity of PI3K with insulin-dependence for growth and survival. It is anticipated that upregulation of PI3K signalling in response to MEK inhibition is a significant factor in the insulin-independent growth observed in 2i and that such a phenotype would not be observed in *Fgf4*-/- ES cells or in 3i where PKB activity is not upregulated. These experiments will be addressed in the near future.

Several attempts were made to directly investigate the role of the PI3K pathway in ES cell self-renewal. The PI3K inhibitor, LY294002, and the PKB-specific inhibitor, AKT-I1/2, both killed ES cells rapidly in serum-free conditions and cultures could not be maintained in their presence. Differentiation was not evident in the cultures but the degree of cell death made it impossible to interpret the effects of these inhibitors. As a further test of the requirement for this pathway I also attempted to culture *Pdk1*-/- ES cells which cannot activate PKB. While these cells were readily maintained in serum-containing media, when transferred to serum-free conditions most cells died and the cultures could not be passaged. Again, although differentiation was not evident, it was impossible to determine whether the cells that were dying would have undergone differentiation. Clearly, investigation of PI3K signalling as a self-renewal signal presents a significant problem. Its established role in anti-apoptotic and pro-proliferative pathways means that its inhibition promotes cell death and it is difficult to assess whether a dead cell will self-renew or commit to differentiation.

3.3.3 Inhibitor Specificity

Establishing the specificity of the pharmacological inhibitors is essential if the mechanisms governing self-renewal are to be elucidated, especially given the heavy reliance on a combination of three for this study. I have established that the major pathway activating ERK1/2 in cells cultured without added cytokines or growth factors is activation of the FGFR by FGF4 produced by the ES cells themselves. In ES cells lacking *Fgf4* neither FGFR inhibitors nor MEK inhibitors were required for propagation of the cells. This clearly indicates that the target of PS is the FGF-MEK-ERK pathway. The possibility that the FGFR inhibitors were required to inhibit another pathway downstream of the FGFR is excluded by the demonstration that a potent MEK inhibitor, PD03, can substitute for PS demonstrating that direct inhibition of the FGFR is not required for efficient self-renewal. This is supported by work on ES cell differentiation which shows that ES cells lacking *Fgf4* or *Erk2* are similarly compromised in their ability to initiate differentiation and that inhibitors of MEK or the FGFR can inhibit ES cell differentiation (Kunath et al., 2007).

In the case of GSK3 cells were made available to us that are null for both isoforms of GSK3. The critical observation is that CHIR has no effect on these cells and that it is no longer required to obtain a 3i-like phenotype. However, the high degree of background differentiation also provided a further test of ERK inhibition. Impressively, it was found that the only conditions that supported self-renewal without a high degree of background differentiation were those in which PS or PD03 were included, largely recapitulating the 3i phenotype. The phenotype of these DKO cells and the abolition of differentiation through ERK1/2 inhibition, as observed through morphological criteria and the striking elimination of Gata4-positive cells, illustrate the potent anti-differentiation effect exerted by PS and PD03. No increase in cell death was evident in these cultures implying that differentiation was inhibited as opposed to the selective elimination of differentiated cells from the cultures. These observations are consistent with recent work showing that ERK signalling is required for ES cells to differentiate into both neural and non-neural lineages (Kunath et al., 2007). However, DKO cells appeared to be compromised in their growth even when differentiation was eliminated and I found that addition of LIF was required to fully restore proliferation. This in itself indicates that LIF can function through parallel pathways to exert effects independently of GSK3.

3.3.4 ES cells cultured in 3i are LIF- and BMP-independent

The dogma of ES cell biology is that ES cells rely on exogenous signals for the activation of pathways that promote their self-renewal. The best characterised of those pathways is LIF-STAT3 which supports self-renewal in the presence of signals that inhibit neural differentiation, provided by serum or BMP4. In examining novel culture conditions it is important to investigate the requirement for these signalling pathways even though the cytokines have not themselves been included in the media. During the development of 3i it was shown that BMP4 was not required if PD18 or SU was added to LIF-containing media (Ying et al, in press). Again this is consistent with the requirement for FGF-ERK signalling for neural differentiation of ES cells (Kunath et al., 2007; Stavridis et al., 2007). In 3i *Id* gene expression was much lower

than in LIF and BMP4 while the addition of PD18 or SU to LIF-containing media did not increase Id1 protein expression above background levels where neural differentiation proceeds efficiently. I conclude that ERK blockade relieves ES cells from their reliance on BMP4 through mechanisms independent of Id expression. During these experiments the complementary question was also addressed: does BMP4 act through inhibition of ERK signalling as has been suggested (Qi et al., 2004)? No evidence was found that BMP4 affects the activity of ERK, in agreement with previous work (Ying et al., 2003a). Furthermore, while the effect of BMP4 on self-renewal appears to be specific to inhibition of neural commitment, ERK inhibition appears to have a more universal effect, imposing a block on differentiation that is not lineage specific (Kunath et al., 2007).

Independence from STAT3 signalling has never been demonstrated for mouse ES cell self-renewal despite numerous publications implicating alternative signalling pathways (Paling et al., 2004; Sato et al., 2004). In this chapter it was demonstrated that STAT3 is not phosphorylated on Tyr705, a site believed to be required for its activation (Ying et al, in press), and that STAT3 target genes are not activated in 3i. However, conclusive proof that self-renewal can proceed independently of STAT3 requires that it is eliminated genetically. Mice heterozygous for a *Stat3*-null allele were crossed, embryos lacking *Stat3* isolated, and ES cells lacking *Stat3* derived. The capacity for self-renewal and differentiation was tested and the cells behaved as ES cells but as expected they could not self-renew in standard culture conditions that are dependent on a LIF-STAT3 signal (Niwa et al., 1998). The prospect of a novel, STAT3-independent mechanism of self-renewal is exciting because the only physiological relevance of STAT3 is its requirement during diapause (Nichols et al., 2001), a phenomenon that does not occur in humans. The hope is that by further understanding the mechanisms of self-renewal in 3i our findings might be generalised to other mammalian species and ES cells derived that can be cultured under similar conditions.

Chapter 4. Is blockade of the ERK1/2 signalling pathway the critical requirement for self-renewal?

4.1 Introduction

ES cells were originally derived by culturing isolated ICMs or delayed implantation blastocysts on a feeder layer in serum-containing media (Evans and Kaufman, 1981; Martin, 1981). Similar conditions are still in routine use by many groups today. Both feeders and serum are sources of undefined signals. In 1988 it was demonstrated that LIF could replace the requirement for feeders (Smith et al., 1988; Williams et al., 1988) but not until 2003 were culture conditions developed that did not require the use of serum – serum was replaced by BMP4 (Ying et al., 2003a). So, in the 27 years or so since ES cells were established they have always been grown in the presence of cytokines and growth factors and often in the presence of sources of undefined signals. It is therefore generally accepted that self-renewal is instructed by extrinsic signals (Cartwright et al., 2005b; Li et al., 2005).

It is now well documented that blockade of the FGF-MEK-ERK signalling cascade promotes self-renewal (Cheng et al., 1998; Qu and Feng, 1998; Burdon et al., 1999b). In fact, in defined culture conditions deletion of *Fgf4* or pharmacological inhibition of downstream signalling components prevents differentiation of ES cells (Kunath et al., 2007; Stavridis et al., 2007). Since there were several means available to us to suppress the differentiation of ES cells I was in a position to assess the nature of ES cells cultured in the absence of extrinsic growth factors.

4.1.1 ERK Signalling in Mouse ES Cells

Activation of receptor tyrosine kinases by their ligands results in autophosphorylation on tyrosine residues on the intracellular domain of the receptor (reviewed in (Yarden and Ullrich, 1988)). These serve as binding sites for SH2 domain-containing proteins including Shp2 and Grb2. Grb2 may directly bind the

receptor and recruit Sos but it can also be recruited to the receptor by Shp2. Localisation of Sos to the membrane brings it into contact with Ras. Ras in turn becomes activated initiating a cascade of phosphorylation that results in activation of ERK1/2 (Fig3.1). ES cells deficient for *Shp2* do not differentiate efficiently into cardiac cell types. They also show a higher efficiency of secondary EB formation (Qu and Feng, 1998), consistent with the idea that *Shp2*-null ES cells in primary EBs are refractory to differentiation. Similarly, *Grb2*-null ES cells do not differentiate into endodermal cell types in EB or adherent differentiation conditions (Cheng et al., 1998). The phenotype can be rescued by expression of Grb2 or by a fusion protein consisting of a C-terminal truncated Sos fused to the SH2 domain of Grb2. Mutation of the SH2 domain abolished the ability of this construct to rescue differentiation implying that interaction with the receptor was required for activation. These experiments clearly demonstrate that Grb2-Sos signalling promotes endodermal differentiation. Furthermore, an activated form of H-Ras restored differentiation implicating the Ras-Raf signalling cascade as the downstream effector.

Consistent with these studies, it has been shown that activation of the MEK-ERK signalling cascade downstream of LIF is dispensable for ES cell propagation (Burdon et al., 1999b). When the Shp2 binding site was mutated on a chimeric gp130 receptor activation of the receptor resulted in STAT3 activation but not activation of ERK1/2. Not only was this receptor able to support self-renewal, efficiency was enhanced in the absence of the ERK signal. This finding could be confirmed by the use of a pharmacological inhibitor of MEK, PD98059, which promoted self-renewal and blocked differentiation in EBs.

A subsequent study focused on the role of ERK signalling in differentiation under defined conditions confirmed that inhibition of the FGF-MEK-ERK signalling pathway biochemically or through genetic manipulation prevents efficient differentiation. ES cells lacking *Fgf4* or treated with an FGFR-inhibitor, and with a corresponding reduction in ERK activation, failed to upregulate the early neural differentiation markers Sox1 (Stavridis et al., 2007) and Nestin (Kunath et al., 2007) and only sporadic βIII-Tubulin-positive neurons could be detected after 10 days'

differentiation (Kunath et al., 2007). Importantly, the phenotype in *Fgf4*-null ES cells could be rescued by the addition of recombinant FGF4, proving that the resistance to differentiation was a result of the loss of FGF4 expression. Strikingly, the same cells were resistant to differentiation into mesoderm and into the large flattened cells that result from BMP4 treatment (Kunath et al., 2007). This indicates that the lack of an FGF signal compromises differentiation along multiple lineages and is not exclusively associated with differentiation into neural lineages. ERK signalling may therefore act as a trigger for differentiation, pushing cells into a state where they are competent to respond to differentiation cues (Fig4.5). This hypothesis is supported by the observation that neural commitment is blocked by the MEK inhibitor PD184352 but removal of the MEK inhibitor for just three hours is sufficient to statistically increase the degree of subsequent differentiation while 9 hours without inhibitor fully restores differentiation efficiency (Stavridis et al., 2007). It appears that a discrete period of ERK activity is sufficient to initiate differentiation.

Taken together the findings outlined above lead to the hypothesis that ES cell self-renewal may be ERK-independent. Moreover, the evidence strongly implicates ERK signalling in the positive regulation of differentiation. Although the studies of *Shp2*-null (Qu and Feng, 1998) and *Grb2*-null (Cheng et al., 1998) ES cells report lineage-specific defects in differentiation the nature of differentiation observed is limited by the culture conditions used and the markers of differentiation examined. These cells may exhibit similarly compromised differentiation to *Fgf4*-null ES cells (Kunath et al., 2007) if examined under conditions designed to promote differentiation along specific lineages (Nishikawa et al., 1998; Ying et al., 2003b). ERK signalling is clearly active under self-renewing conditions as it is activated by LIF (Burdon et al., 1999b) and by autocrine FGF4 (Kunath et al., 2007) and is readily detected in self-renewing ES cells by immunoblotting (see chapter 3). Therefore, ERK signalling is required but not sufficient to drive differentiation, which is prevented in standard culture conditions by activation of STAT3 (Niwa et al., 1998; Matsuda et al., 1999) and serum or BMP-induced expression of Id genes (Ying et al., 2003a). In chapter 3 it was demonstrated that in 3i ES cells are relieved from their dependence on STAT3 and BMP signalling. In this chapter the question of whether ES cells require any

further signalling input to self-renew when differentiation is prevented by inhibition of the ERK1/2 signal is addressed.

4.2 Results

4.2.1 Propagation of ES cells in PD184352 plus SU5402 (PS) or PD0325901

ES cells cultured in the presence of a MEK inhibitor or an FGFR inhibitor retain expression of pluripotency-associated genes under conditions where they would otherwise be expected to differentiate (Ying et al., 2003b; Kunath et al., 2007; Stavridis et al., 2007). In chapter three I showed that a combination of PD18 and SU (PS) reduced ERK phosphorylation at concentrations where the individual inhibitors had little effect. A more potent MEK-inhibitor, PD03, was also tested and substituted effectively for PS at 1μM, providing a near-total block of ERK phosphorylation at this concentration. To examine the effect of ERK inhibition I attempted to culture ES cells in PS alone and later PD03 alone.

To grow cells in PS alone it was important to first culture the cells in serum-free conditions for 1-2 passages and to expose the cells to PS alone only after they had been allowed to attach to the plate in the presence of LIF or CHIR. Passaging cells directly from LIF- or CHIR-containing conditions into media containing PS alone results in a large degree of cell death and failure of the passaged cells to adhere to gelatine. However, once cells are established in PS as described above they can be maintained in culture for more than one month and 8 passages (Fig4.1a). ES cells grown in PS alone proliferated slowly and exhibited poor viability with cultures containing a large number of floating, dead cells. The cells had a doubling time at least twice that of side-by-side cultures in LIF and BMP4 or 3i and as a result were passaged at relatively high density. With the exception of a few poor colonies in colony forming assays it was not possible to obtain clonal propagation of ES cells in

PS or PD03 alone, suggesting that their survival might be density-dependent. Although long-term culture in PS or PD03 was demonstrated for multiple independent ES cell lines (Oct4GIP, E14Tg2a and 46C (Q-L. Ying, unpublished)) the failure to support clonal propagation means that the conditions do not pass one of the 'tests' normally applied to demonstrate sufficiency of any culture condition to support self-renewal. There are several possible reasons for the insufficiency of these conditions. If differentiation is incompletely blocked, either because ERK retains some activity or because a degree of differentiation can proceed in the absence of ERK signalling then cells at low density may become more vulnerable and fail to establish undifferentiated colonies. Alternatively, differentiation may be effectively blocked but the cells are otherwise compromised in terms of their viability or growth and proliferation.

In PS cultures a degree of spontaneous differentiation was observed, including the appearance of rosette-like structures indicative of neural differentiation (Fig4.1b). This neural differentiation can be inhibited by the addition of BMP4 (10ng/ml) but efficient self-renewal is not restored. Rather, the neural rosettes are replaced by flat sheets of cells (Fig4.1b,c) typical of BMP4-induced differentiation (Ying et al., 2003a). Notably, however, cultures in PS plus BMP4 can be maintained for at least 7 passages (Fig4.1b,c) unlike cultures in BMP4 alone which cannot be passaged (Ying et al., 2003a) indicating that PS partly blocks the potent differentiation-inducing activity of BMP4. This is an important point; the block in differentiation imposed by PS is not specific to neural lineages, in agreement with recently published work (Kunath et al., 2007). When late-passage PS cultures were immunostained for Oct4 there were a significant number of Oct4 negative cells (Fig4.1a) indicating heterogeneity in the culture. Analysis of gene expression by real-time PCR showed that the primitive ectoderm marker, *Fgf5,* and the early neural marker, *Nestin*, were upregulated in PS relative to LIF and BMP4 or 3i cultures (Fig4.1d) implying that the cultures contain a proportion of cells undergoing differentiation. I conclude therefore that differentiation it not completely blocked in PS. In an attempt to establish if this results from residual ERK activity ES cells were cultured in

relatively high concentrations of PD03. At 1-2μM pERK is barely detectable by immunoblotting (see chapter 3). However, ES cells cultured in 1-2μM PD03 proliferated poorly and could not be maintained beyond passage 4. The failure to expand cells further in PD03 alone could result from toxic effects of the inhibitor but may also reflect a requirement for low levels of ERK activity for continued growth or proliferation. However, the apparent heterogeneity of cultures in PS suggests that incomplete inhibition of ERK activity may permit some differentiation to proceed.

Addition of LIF or CHIR to PS fully restores self-renewal with minimal differentiation evident (Fig4.1e, note flattened morphology as compared to rounded up colonies formed in presence of LIF or CHIR respectively). When single cells are plated into individual wells of a 96-well plate the efficiency of colony formation in 3i or LIF plus PS is similar to that observed in LIF plus BMP4 (Fig4.1f). In fact, CHIR could fully rescue propagation in 2μM PD03 (Fig4.1g) even in clonal assays using an ES cell line subcloned from E14Tg2a (E14 IV C) (Fig4.1h) and recently tested for germ-line transmission, suggesting that ERK activity is fully dispensable if viability and/or proliferation are promoted by the addition of LIF or CHIR. Both LIF and CHIR are predicted to have pleiotropic effects on ES cells with potential roles in inhibition of differentiation and regulation of cell metabolism and the cell cycle. It is therefore difficult to distinguish the ‘essential’ contributions of these molecules from effects that are beneficial for ES cell culture but not strictly required to instruct cell fate decisions in favour of self-renewal over differentiation.

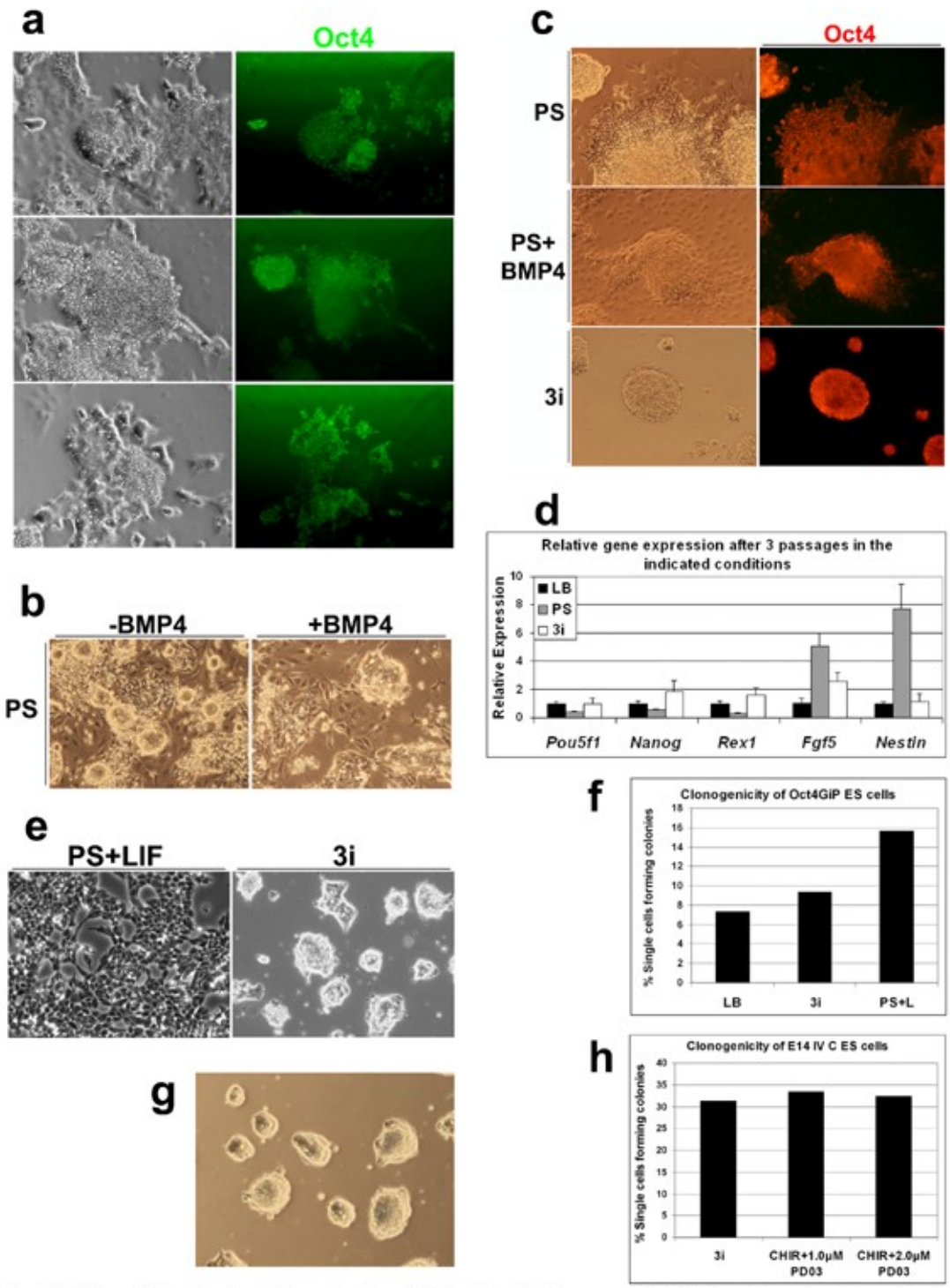


Fig4.1. PS partially blocks differentiation of ES cells. a) 3 images of E14Tg2a ES cells cultured in PS for 8 passages. Cells were fixed and immunostained for Oct4. b) Phase contrast images of E14Tg2a ES cells cultured in PS with or without BMP4. c) E14Tg2a ES cells cultured for 7 passages in PS with or without BMP4 or in 3i were fixed and immunostained for Oct4. d) Histogram showing real-time PCR analysis of marker expression in E14Tg2a ES cells after 3 passages in LIF and BMP4 (LB), PS or 3i. e) Phase contrast images of E14Tg2a ES cells cultured in PS+LIF or 3i. f) Histogram showing percentage of single Oct4GIP cells forming ES colonies in LIF and BMP4 (LB), 3i or PS plus LIF (PS+L). g) Image of E14Tg2a ES cells cultured in 3µM CHIR plus 2µM PD03. h) Histogram showing percentage of single E14 IV C ES cells forming ES colonies in 3i or 3µM CHIR plus 1 or 2µM PD03.

4.2.2 Inhibition of apoptosis by forced expression of *Bcl2* facilitates the examination of self-renewal in PS.

The phenotype of ES cells cultured in PS or PD03 alone became difficult to assess. While differentiation was largely inhibited cell viability was poor. The failure to form colonies at low density could have resulted from differentiation, cell death or proliferation arrest. The effect of LIF and CHIR could be to inhibit residual differentiation, to increase cell viability or proliferation, or a combination of these effects. In an attempt to separate the role for anti-apoptotic signals from instructive signals in cell fate decisions the effect of overexpressing *Bcl2*, an anti-apoptotic gene (reviewed in (Zinkel et al., 2006)) was assessed. It was predicted that in PS cultures, where cell death is high, inhibition of apoptosis would reveal the fate of the dying cells. If ES cells themselves were undergoing apoptosis then self-renewal would be improved by inhibition of apoptosis whereas if the cell death reflected apoptosis in cells that had committed to differentiate, their terminally differentiated products would be observed in the culture.

Bcl2 expression is ordinarily expressed at low but detectable levels in ES cell populations. *Bcl2*-coding sequence was amplified by PCR from total ES cell cDNA and subcloned into an expression vector. This vector drives *Bcl2* expression from a constitutively active CAG promoter and can be selected for by the addition of puromycin as a puromycin-resistance cassette is transcribed from the same promoter and translated from an internal ribosome entry site (IRES). ES cells stably expressing *Bcl2* (Bcl2-ES) were isolated following electroporation of linearised plasmid DNA and selection for puromycin resistance. As a control, equivalent amounts of the vector plasmid were transfected into a separate pool of ES cells and selected. In side-by-side experiments Bcl2- and vector-transfected ES cells were cultured in N2B27 alone, PS or 3i. After 10 days of selection the plates were fixed and stained for alkaline-phosphatase to identify undifferentiated colonies. In N2B27 alone, colonies failed to form with either plasmid while in 3i undifferentiated colonies formed efficiently in both cases. In PS however, significantly more colonies formed in Bcl2-transfected cells than in the case of the vector (Fig4.2a). This implies that preventing apoptosis does permit colony formation in PS. Note however that these conditions do

not represent expansion of colonies from isolated cells as the initial plating density is high. Only after elimination of a large proportion of the ES cells by selection do colonies appear that are assumed to have arisen from a single, stably transfected cell. Clones were expanded from duplicate plates for further analysis.

To assess the level of apoptosis in PS cultures and the effect of forced Bcl2 expression the loss of membrane asymmetry was examined using an AnnexinV assay. Co-staining with propidium iodide (PI) allowed dead cells to be excluded. Apoptotic cells are defined as those cells which are PI negative (live) but AnnexinV positive (apoptotic) (Fig4.2b). Note that neither PS nor PD03 increased apoptosis relative to N2B27 alone indicating that inhibition of ERK signalling does not result in increased apoptosis. Cultures of wild-type ES cells in PS alone had a higher proportion of apoptotic cells than cultures containing LIF or CHIR (Fig4.2b,c). LIF and CHIR can therefore inhibit apoptosis either directly or indirectly. As expected, Bcl2-ES cells exhibited decreased apoptosis in all conditions examined (Fig4.2b,d). As Bcl2 was apparently functioning to inhibit apoptosis in ES cells it was possible to examine the effect of this on ES cell self-renewal in different culture conditions.

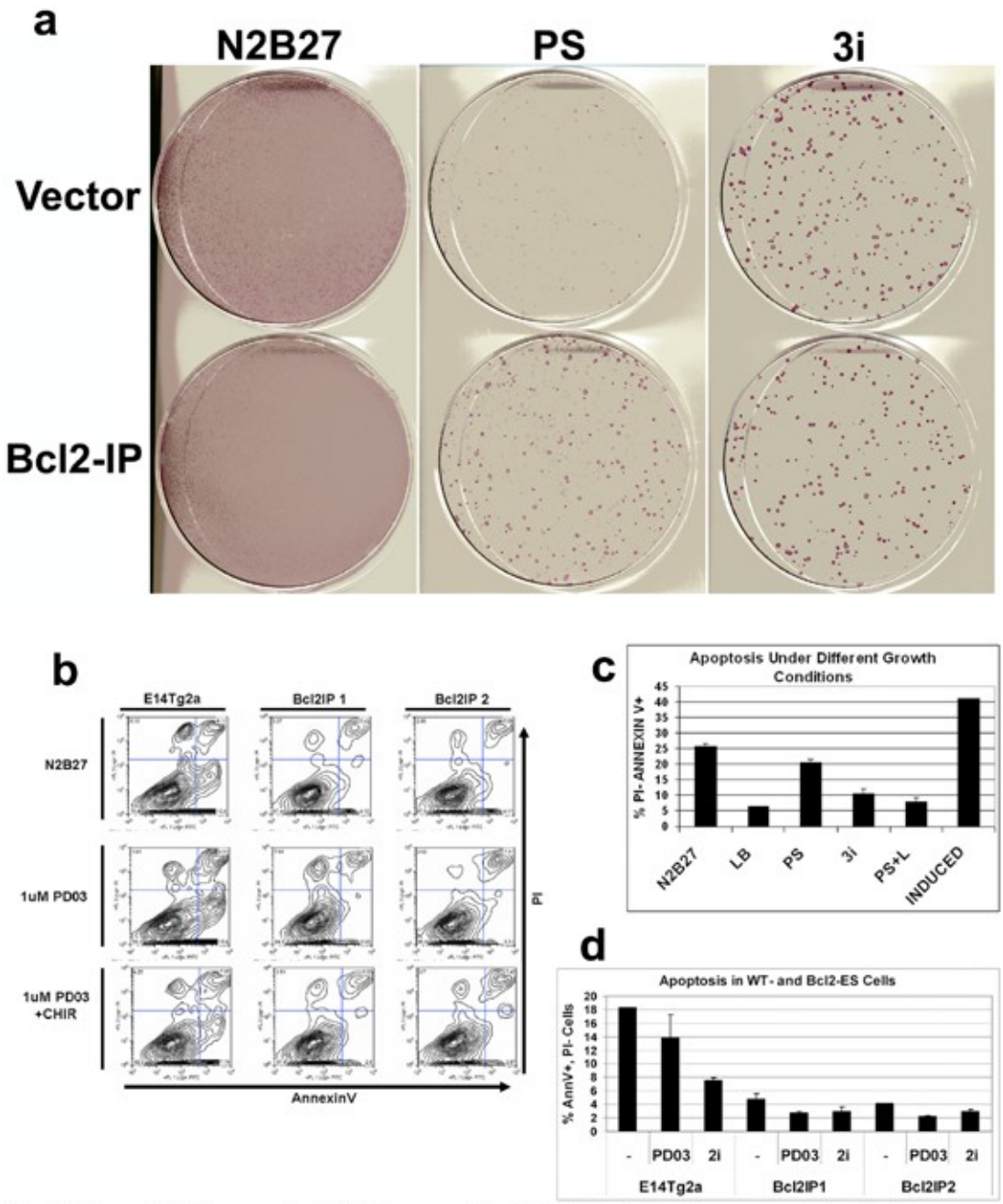


Fig4.2. Forced Bcl2 expression inhibits apoptosis. a) E14Tg2a ES cells were transfected with linearised plasmid DNA encoding Bcl2 (Bcl2-IP) or the vector plasmid and cells carrying stable integrations isolated by selecting for puromycin resistance in N2B27 alone or plus PS or 3i. After 10 days' selection plates were fixed and stained for alkaline phosphatase. b) Contour plots showing the profile of AnnexinV and PI staining in parental (E14Tg2a) or Bcl2-expressing (Bcl2IP1/2) ES cells grown in N2B27 alone or plus 1μM PD0325901 (PD03) or 1μM PD03 and 3μM CHIRON99021 (CHIR). Apoptotic cells are defined as PI-negative, AnnexinV positive (lower right quadrant) c) Histogram showing percentage apoptotic cells in N2B27 alone or plus LIF and BMP4 (LB); PS; 3i; or PS + LIF (PS+L). "Induced" indicates cells treated with camptothecin for 4 hours to induce apoptosis. Error bars represent standard deviation of the mean for 2 biological replicates. d) Histogram showing percentage apoptotic cells in parental or Bcl2-expressing ES cells after 48hrs' culture in N2B27 alone (-) or plus 1μM PD03 (PD03) or 1μM PD03 and 3μM CHIR (2i). Error bars represent standard deviation of the mean for 2 biological replicates.

4.2.3 Bcl2 does not inhibit differentiation of ES cells

The use of Bcl2 to inhibit apoptosis presented a potential problem. Bcl2 has been reported to support self-renewal of ES cells in the presence of LIF and the absence of serum (Yamane et al., 2005). It has previously been shown that cells cultured in LIF alone undergo neural differentiation which can be inhibited by the addition of BMP4 or serum (Ying et al., 2003a). The finding that Bcl2 relieves ES cells from their dependence on BMP4 or serum implies that it can prevent neural differentiation. For forced Bcl2 expression to be a useful tool for our analyses it was important that it did not inhibit differentiation. To examine this, Bcl2-ES cells were cultured in LIF alone. Culture in N2B27 in the absence of LIF served as a positive control for neural differentiation while addition of PD03, which blocks differentiation, served as a negative control. Cultures were fixed and stained on the 2nd passage in these conditions. Survival in the parental cell line was poor in N2B27 alone so no cells survived to the 2nd passage for staining. Neural differentiation was judged by the appearance of β-III-Tubulin positive cells (reactive to the Tuj1 antibody) with distinct neuronal morphology. Efficient neural differentiation was observed in N2B27 alone as expected and also in the presence of LIF although ES cells persisted for longer when LIF was added to the media as judged by the persistence of Nanog-positive cells (Fig4.3a). If anything, the expression of Bcl2 appeared to enhance differentiation (Fig4.3a, compare E14Tg2a to Bcl2-ES cells in LIF alone), probably by decreasing the amount of cell death normally seen upon monolayer neural differentiation (Ying et al., 2003b). Differentiation in LIF-containing conditions was almost totally eliminated by the addition of 1μM PD03 (Fig4.3a) with only very rare Tuj1-reactive cells detected. Confident that Bcl2 does not block neural commitment the growth of Bcl2-ES cells in PS or PD03 alone was assessed.

Wild-type ES cells can be maintained in PS but cell death is high and there is evidence of heterogeneity (Fig4.1). Bcl2-ES cells exhibit reduced cell death in PS as expected. Bcl2-ES cells were cultured in PS, PD03 or 3i for several passages, plating onto laminin for the purposes of staining on the final passage (increased substrate attachment results in the flattened morphology observed in 3i, Fig4.3b). Mature

neurons emerge in PS culture conditions (Fig4.3b) indicating that neural differentiation is proceeding even with the reduced levels of ERK signalling in PS. Neural differentiation was completely abrogated by the addition of CHIR (Fig4.3b, 3i). 3i-cultured Bcl2-ES cells appeared homogeneous with virtually all cells retaining expression of Oct4 (Fig4.3b). This indicates that the high proportion of ES cells in 3i is unlikely to result from selection in favour of ES cells over their differentiated progeny as differentiated cells would be expected to be evident in Bcl2-ES cultures if differentiation was occurring. This is consistent with the low levels of cell death seen in established 3i cultures.

To determine if the neural differentiation observed in PS cultures could be eliminated by further reduction of the ERK signal Bcl2-ES cells were cultured in 1 or 2μM PD03. Unlike WT-ES cells Bcl2-ES cells grew in the higher concentrations of PD03 implying that Bcl2 expression rescues them from apoptosis induced by greater MEK inhibition or non-specific effects of the inhibitor. A substantial decrease in the number of Tuj1-reactive neurons formed (Fig4.3b,c) as compared to PS conditions was observed but neural differentiation was not entirely eliminated indicating that either the ERK signal was not totally eliminated or that some differentiation proceeds in the absence of ERK activity. It is worth noting here that our assays of ERK activity can only be as sensitive as immunoblotting permits and that ERK activity is assessed in the population, not at the level of individual cells where variation may still exist. It is not possible from these studies to conclude that individual cells have succeeded in entering the differentiation program without activating ERK. From these experiments claims can be made only about how the population behaves in response to the inhibitors and how this correlates with global levels of ERK activation.

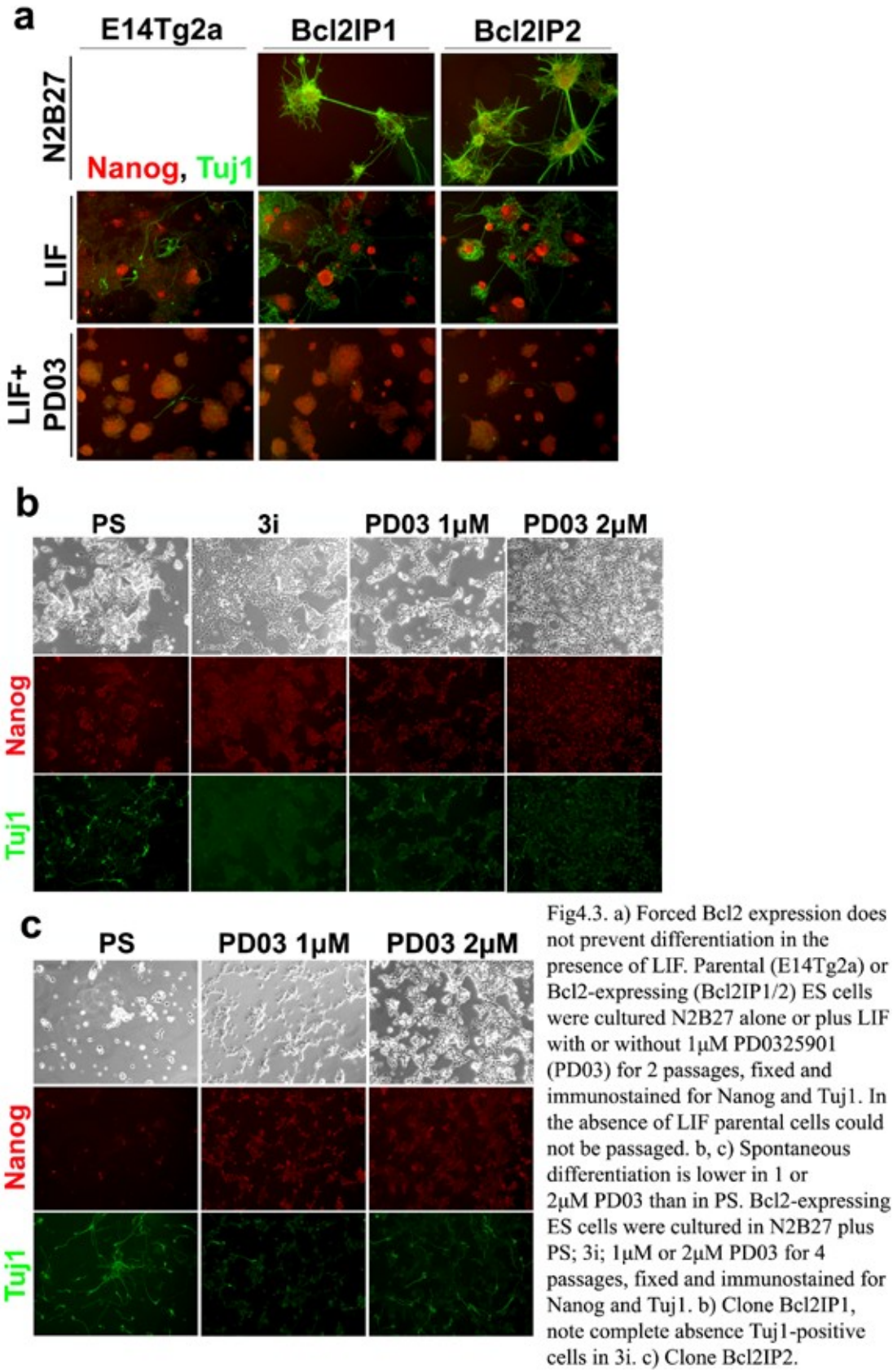


Fig4.3. a) Forced Bcl2 expression does not prevent differentiation in the presence of LIF. Parental (E14Tg2a) or Bcl2-expressing (Bcl2IP1/2) ES cells were cultured N2B27 alone or plus LIF with or without 1μM PD0325901 (PD03) for 2 passages, fixed and immunostained for Nanog and Tuj1. In the absence of LIF parental cells could not be passaged. b, c) Spontaneous differentiation is lower in 1 or 2μM PD03 than in PS. Bcl2-expressing ES cells were cultured in N2B27 plus PS; 3i; 1μM or 2μM PD03 for 4 passages, fixed and immunostained for Nanog and Tuj1. b) Clone Bcl2IP1, note complete absence Tuj1-positive cells in 3i. c) Clone Bcl2IP2.

Given the reduction of differentiation in PD03 the cultures were maintained long-term. The cells were readily passaged and grew well albeit less quickly than LIF- or CHIR-containing cultures. After 8 passages and more than a month in continuous culture the cells were stained for markers of pluripotency, Oct4 and Nanog (Fig4.3.1a). A significant proportion of the remaining cells were Oct4- and Nanog-negative (Fig4.3.1a,b) indicating that the population was heterogeneous and that despite the apparent efficiency of long-term culture the combined effects of ERK inhibition and anti-apoptotic signals did not reproduce the homogeneous ES cell growth seen in 3i or in standard LIF-containing culture conditions. The immunostaining results are supported by real-time PCR analysis showing that *Oct4* and *Nanog* are approximately 2-fold down-regulated while *Nestin* is significantly upregulated and *Gata4* slightly, but reproducibly upregulated (Fig4.3.1c). Interestingly, increasing the dose of PD03 from 1μM to 2μM did reduce the level of *Nestin* detected in the population (Fig4.3.1c), suggesting that there may be a further reduction in differentiation resulting from increased MEK inhibition.

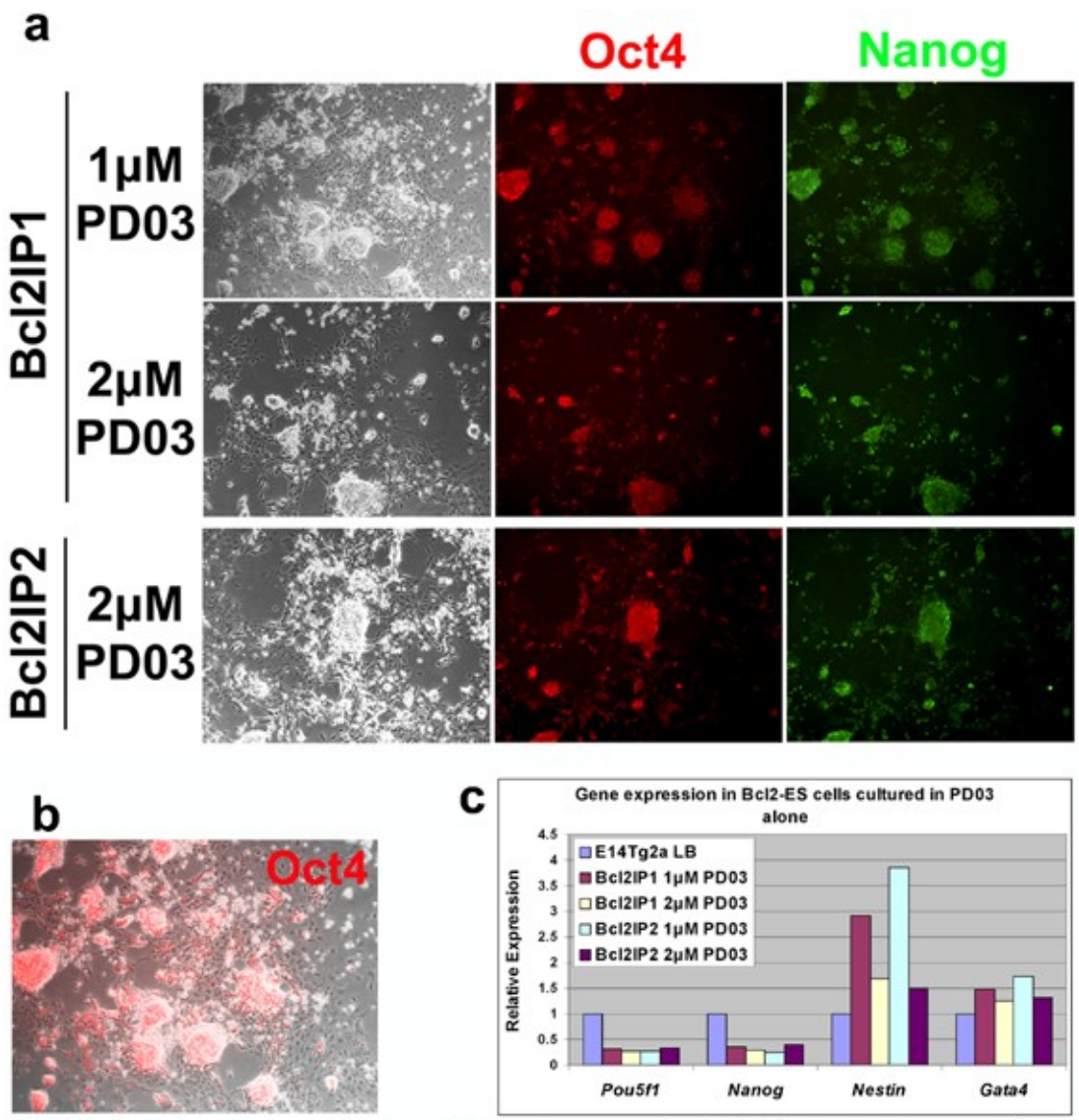


Fig4.3.1. Forced expression of Bcl2 permits long-term expansion in PD03 alone but the population is heterogeneous. a) After 8 passages in 1 or 2μM PD0325901 (PD03) Bcl2-expressing ES cells (Bcl2IP1/2) were fixed and immunostained for Nanog and Oct4. b) Overlay of phase contrast and Oct4 immunofluorescence images from (a), note significant proportion of Oct4-negative cells. c) Histogram showing real-time PCR analysis of marker expression in Bcl2IP1/2 cells after 8 passages in N2B27 plus 1 or 2μM PD03. Gene expression was normalized to β-actin for each sample and normalised values compared to gene expression in parental (E14Tg2a) ES cells cultured in N2B27 plus LIF and BMP4 (LB).

4.2.4 Nanog

Nanog is one of three TFs essential for the establishment of a pluripotent epiblast (Mitsui et al., 2003) and required for the efficient self-renewal of ES cells (Mitsui et al., 2003; Chambers et al., 2007). Nanog overexpression permits self-renewal in the

absence of LIF and BMP4 (Chambers et al., 2003) demonstrating that it is capable of suppressing differentiation. It has recently been shown that Nanog expression is not homogeneous in self-renewing ES cells (Chambers et al., 2007). Cells can be identified that retain the expression of Oct4 but express little or no Nanog. Importantly, these cells can reexpress Nanog as demonstrated by the isolation of the Nanog-negative population and the subsequent emergence of a Nanog positive population from those cells. The Nanog-negative population are however more prone to differentiation suggesting that elevated Nanog levels do enhance self-renewal. The implication of these studies is that culture conditions promoting elevated levels of Nanog expression or increased homogeneity of Nanog expression within the population should increase the efficiency of self-renewal.

The phenotype of ES cells cultured in PS or PD03 is similar to that observed for Nanog-overexpressing cells (Chambers et al., 2003). They are compromised in their ability to differentiate and self-renewal is enhanced in response to LIF or CHIR. Notably however, Nanog-overexpressing cells retain the ability to form colonies at clonal density, unlike wild-type cells in PS or PD03 (Fig4.4g). I hypothesized that the phenotype of 3i culture could result, at least in part, from regulation of Nanog expression and in particular that the inhibition of differentiation resulting from ERK inhibition might be mediated by elevated levels of Nanog expression.

Regulation of Nanog has been linked to the MEK-ERK pathway. One group showed that treatment of ES cells with sodium vanadate, a tyrosine phosphatase inhibitor, results in Nanog down-regulation and differentiation into primitive endoderm (Hamazaki et al., 2006). This does not occur in the absence of Grb2 and is inhibited by the MEK inhibitor, PD98059, implicating receptor tyrosine kinase coupling through Grb2 to activate the MEK-ERK signalling pathway in the down-regulation of Nanog. However, this study provides no evidence that Nanog is a direct downstream target of ERK signalling. In the preimplantation embryo there is also evidence of Grb2-mediated regulation of Nanog. In *Grb2*-null blastocysts all of the ICM cells express Nanog at a point when wild-type embryos show ‘salt and pepper’ expression of Nanog and Gata6 and subsequently a clear segregation of the Nanog

positive epiblast from the Gata6 positive primitive endoderm (Chazaud et al., 2006). This phenotype is consistent with the hypothesis that signalling downstream of Grb2 results in Nanog down-regulation and Gata6 upregulation although no mechanism has been proposed for this phenotype and it is not known if or how signalling through Grb2 is activated in some cells but not others in the ICM. The phenotype of *Grb2*-null blastocysts can be reproduced by culturing embryos from the 8-cell stage, prior to primitive endoderm specification, in the presence of PS or PD03. Embryos developing in the presence of these inhibitors homogeneously express Nanog in all cells of the ICM and fail to form the Gata4-positive primitive endoderm (J. Silva and J. Nichols, unpublished). This appears to result from an expansion of the epiblast at the expense of primitive endoderm as the total cell number in the embryo is similar and the total cell number in the ICM increased in the presence of the inhibitors. Furthermore, when blastocysts are cultured in the presence of inhibitors after the primitive endoderm has formed, the primitive endoderm does not die indicating that pre-formed primitive endoderm can survive and the inhibitors function to block its specification as opposed to selectively eliminating somatic cells (J. Silva and J. Nichols, unpublished). From these studies I predicted that MEK inhibition would result in upregulation of Nanog in the ES cell population.

Examination of *Nanog* expression in steady-state conditions reveals that *Nanog* mRNA is present at slightly higher levels in 3i than in LIF and BMP4 despite equivalent levels of Oct4 expression (Fig4.1d). Elevated levels of expression are consistent with increased *Nanog* transcription in individual cells or an increase in the proportion of cells in the population expressing *Nanog*. Steady-state expression levels are subject to changes in cell phenotype and do not therefore indicate direct regulation. The direct response of *Nanog* to short term treatment with the inhibitors was examined. ES cells were starved of serum and cytokines and then stimulated for 1 hour with LIF or PS. LIF served as a positive control for activation of the ERK target *Egr1* but had no effect on *Nanog* expression. Neither were *Nanog* transcript levels affected by MEK inhibition despite clear down-regulation of *Egr1* (Fig4.4a), suggesting that *Nanog* transcription is not directly regulated by ERK activity.

Nanog protein levels were examined next. When cultured in N2B27 alone, Nanog is substantially down-regulated after just 24 hours in culture (Fig4.4b). The degree of Nanog down-regulation is reduced in the presence of MEK inhibitor or FGFR inhibitor and the two inhibitors combined in PS further support Nanog expression. CHIR alone was also able to maintain Nanog expression during this relatively short term assay, consistent with the undifferentiated phenotype of ES cells after short periods in CHIR alone (see chapter 5). The combination of all three inhibitors, 3i, showed the highest level of Nanog expression after the 24 hour culture period (Fig4.4b) suggesting that the inhibitors work together to upregulate Nanog either directly or indirectly by maintaining a high proportion of undifferentiated cells in the population. To test the direct response of the cells to the inhibitors or to LIF cells were cultured in N2B27 alone for 12 hours and then treated for a further 4 or 8 hours. There was no direct response to either LIF or CHIR but PS clearly induced up-regulation of Nanog protein at both time points (Fig4.4c). No significant change in Oct4 levels was observed indicating that the change in Nanog levels did not reflect an increase in the proportion of ES cells in the population. As this response was observed following just 4 hours' treatment it seems that Nanog expression levels are extremely sensitive to ERK activity. The response of Nanog to PS was not significantly enhanced by the addition of CHIR (Fig4.4c, compare PS and 3i), implying that ERK- but not GSK3-inhibition results in increased Nanog protein levels.

As discussed above, the regulation of Nanog is likely to contribute to the phenotype of ES cell cultures. To assess the importance of Nanog upregulation for 3i culture ES cells from which *Nanog* has been deleted were cultured. *Nanog*-null ES cells have been established and maintained previously in standard ES cell culture conditions (Chambers et al., 2007). They exhibit substantially increased spontaneous differentiation which is effectively eliminated by selecting for antibiotic resistance genes expressed from the *Nanog* locus. In the absence of selection two independently-established *Nanog*-null lines (44Cre6 and BT12) were readily maintained in 3i, 2i or in PD03 plus LIF (Fig4.4d,4.5a). Both lines were maintained for at least one month (8 passages) in culture and retained the expression of

pluripotency markers as assessed by RT-PCR (Fig4.4e) and immunostaining or *Nanog*-GFP expression (44Cre6 has a GFP reporter knock-in in the *Nanog* locus (Chambers et al., 2007)) (Fig4.5a). However, a degree of spontaneous differentiation persisted, particularly in clone 44cre6 (Fig4.4d,4.5a). Our observations indicate that Nanog expression is not essential for the culture of ES cells in 3i but that the absence of Nanog does increase spontaneous differentiation, which is virtually absent from wild-type cultures. The direct effect of the MEK inhibitor can most easily be seen by comparing cultures of 44Cre6 in N2B27 alone or plus PD03. PD03 alone results in persistence of *Nanog*-GFP positive cells while it is completely lost from cells in N2B27 alone within two passages (Fig4.5b). These results indicate that MEK inhibition can inhibit differentiation independently of Nanog expression.

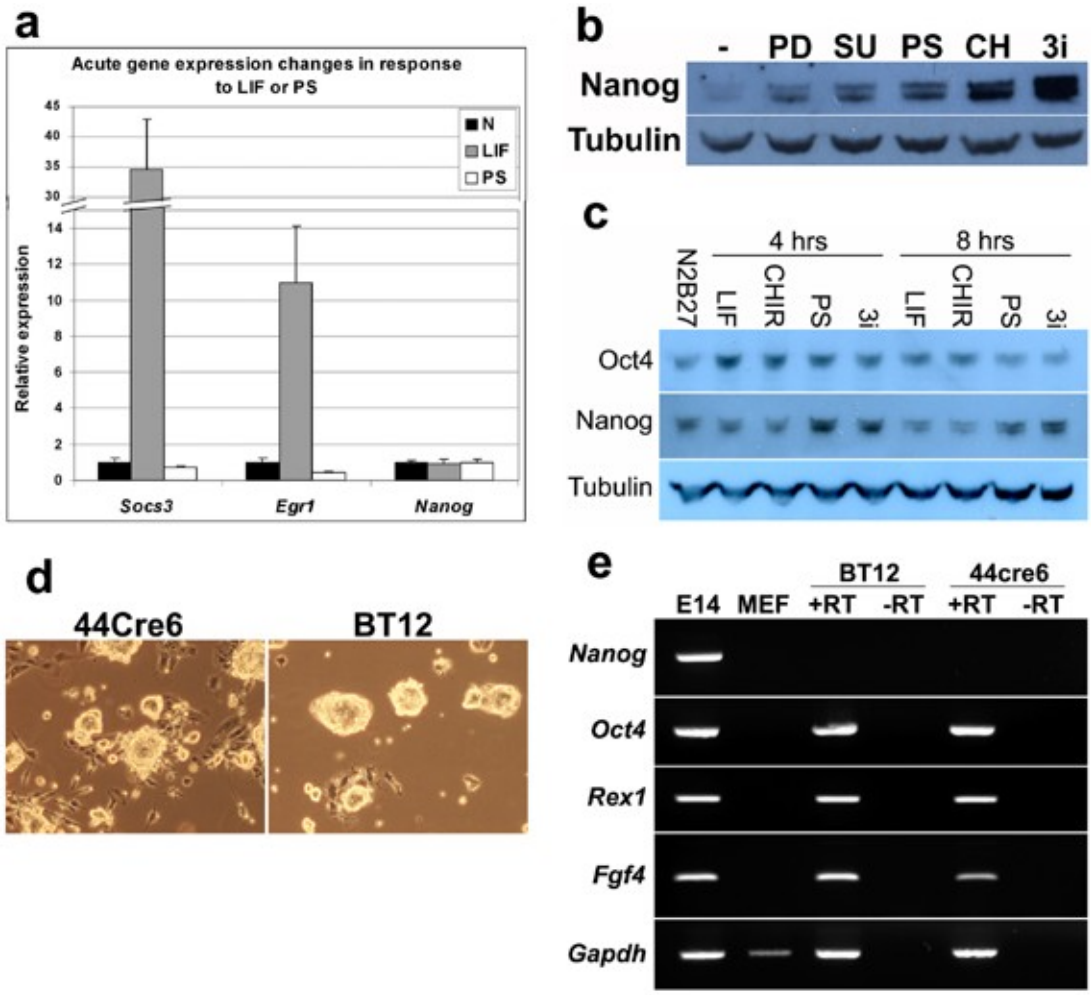


Fig4.4. The role of Nanog in 3i. a) Histogram showing relative gene expression in E14Tg2a ES cells. Cells were grown for 4 hours in N2B27 alone and a further 1 hour in N2B27 alone (N) or plus LIF or PS. Gene expression was normalised to β-actin for each sample and normalised values compared to the unstimulated condition (N). Error bars represent standard deviation of the mean for 3 biological replicates. b) Immunoblot showing Nanog protein levels in E14Tg2a ES cells after 24 hours in N2B27 alone (-) or plus PD184352, 0.8μM (PD); SU5402, 2μM (SU); PS; CHIRON99021, 3μM (CH); or 3i. Tubulin serves as a loading control. c) Immunoblot showing Nanog and Oct4 protein levels relative to Tubulin loading control. E14Tg2a ES cells were cultured for 12 hours in N2B27 alone and a further 4 or 8 hours in N2B27 alone or plus LIF; CHIRON99021, 3μM (CHIR); PS; or 3i before lysis. d) Phase contrast images of *Nanog*-null ES cell lines, 44Cre6 and BT12, after 7 or 6 passages respectively in 2i. e) RT-PCR analysis of pluripotency marker expression in *Nanog*-null ES cells after 8 passages in 2i. E14Tg2a ES cells (E14) and MEFs serve as positive and negative controls respectively. +RT and -RT indicate +/- reverse transcriptase respectively.

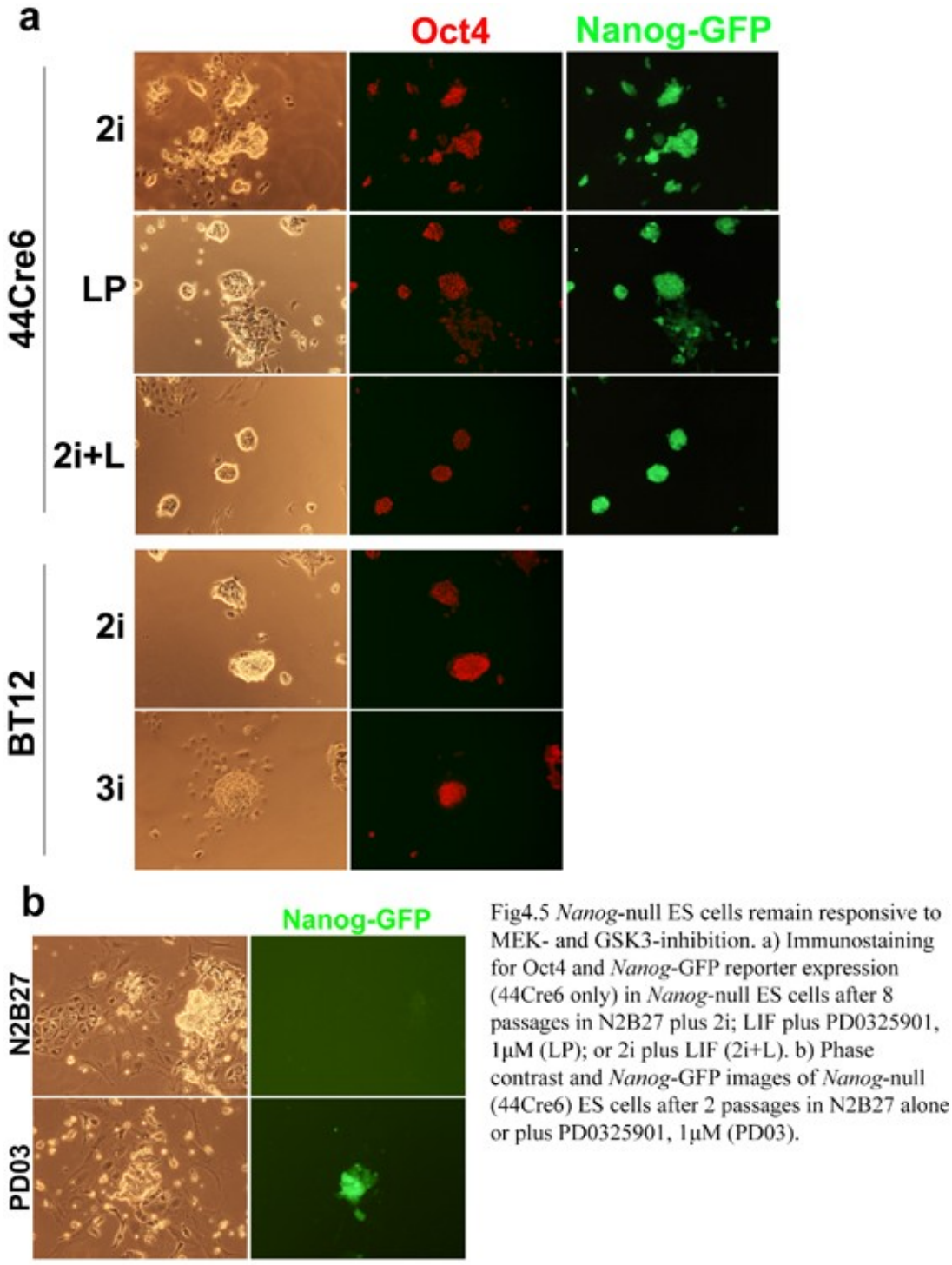


Fig4.5 *Nanog*-null ES cells remain responsive to MEK- and GSK3-inhibition. a) Immunostaining for Oct4 and *Nanog*-GFP reporter expression (44Cre6 only) in *Nanog*-null ES cells after 8 passages in N2B27 plus 2i; LIF plus PD0325901, 1μM (LP); or 2i plus LIF (2i+L). b) Phase contrast and *Nanog*-GFP images of *Nanog*-null (44Cre6) ES cells after 2 passages in N2B27 alone or plus PD0325901, 1μM (PD03).

4.3 Discussion

I found that ES cells could be propagated in N2B27 supplemented with only PS. In the absence of inhibitors ES cells undergo neural differentiation (Ying et al., 2003b)

and cannot be passaged more than once. Propagation over 8 passages and the maintenance of Oct4 expression clearly demonstrates that ERK inhibition has a profound effect, preventing otherwise inevitable differentiation and accompanying deterioration and loss of the cultures. However, the cell population is clearly heterogeneous and clonal propagation is not possible so ERK inhibition cannot be described as sufficient for ES cell self-renewal. The inability to grow from single cells suggests that paracrine signalling might be involved in growth at higher density. It would be interesting to test if ES cells lacking the LIFR or STAT3 (see chapter 3) could be grown in PS. This would at least rule out a role for paracrine LIF signalling.

4.3.1 Inhibition of apoptosis by Bcl2 facilitates the study of cell fate

The use of Bcl2 as an anti-apoptotic agent proved to be informative. It is often difficult to assess the contribution of signalling pathways to self-renewal because their disruption leads to a decrease in viability or proliferation. If the cells become difficult to propagate it is difficult to assess the role of the disrupted pathway in the instruction of cell fate choice. The PI3K pathway is a good example with contrasting reports on the relative contribution to cell cycle (Jirmanova et al., 2002), anti-apoptosis (Sun et al., 1999; Stiles et al., 2002) and inhibition of differentiation (Paling et al., 2004; Watanabe et al., 2006). If cells were prevented from undergoing apoptosis it might become possible to tease out a role for PI3K signalling in cell fate choice. The experiments described here suggest that this might be made possible by Bcl2 overexpression. In the context of PS culture Bcl2 expression resulted in the accumulation of mature neurons revealing that the ES cells were in fact committing to the neural lineage in PS and that the dead cells habitually present in PS cultures are likely to have come from cells that have initiated differentiation and subsequently undergone apoptosis. The death of differentiating cells in PS may result from a requirement for ERK signalling in somatic cell derivatives of pluripotent ES cells.

4.3.2 ERK inhibition prevents differentiation but efficient self-renewal requires signalling downstream of LIF or GSK3

The established role of ERK signalling in the promotion of differentiation (Burdon et al., 1999b) and the demonstration here of our ability to culture cells in PS or in PD03 for an extended period suggest that inhibition of ERK signalling may represent a minimum requirement for self-renewal through the prevention of differentiation. Having removed LIF from the culture conditions it will surprise many that the cells grew at all. However, the high rate of spontaneous differentiation that persists in cultures of ES cells in PS or PD03 alone demonstrates that inhibition of ERK signalling is not sufficient to support efficient self-renewal.

The addition of LIF or CHIR to PS or PD03 eliminates residual differentiation from the cultures. LIF and CHIR may activate signalling pathways that inhibit differentiation independently of ERK activity. This is known to be the case for LIF because it prevents the non-neural differentiation that occurs in the presence of serum or BMP4 (Ying et al., 2003a) despite activating ERK signalling (Burdon et al., 1999b). Wnt/β-catenin signalling has also been implicated in self-renewal even under conditions where ERK is known to be highly active (Sato et al., 2004; Liu et al., 2006). Activation of STAT3 or of β-catenin may therefore promote self-renewal in the context of ERK inhibition by blocking residual differentiation. This effect is most obvious from the elimination of neural differentiation caused by the addition of CHIR to PS. Wnt signalling has previously been reported to inhibit neural differentiation of ES cells (Aubert et al., 2002; Haegele et al., 2003) and this is likely to be replicated by CHIR through stabilisation of β-catenin. However, this explanation does not fully support the observed phenotypes. PS and PD03 largely prevent differentiation and permit long term bulk culture but colonies cannot be obtained at clonal density. If inhibition of differentiation were sufficient for clonal propagation some colonies would be expected to form in PD03 alone. I propose therefore that LIF and CHIR do not simply repress residual differentiation but enhance cell survival and proliferation.

STAT3 is the key signalling molecule downstream of LIF (Niwa et al., 1998; Matsuda et al., 1999). Elevated levels of STAT3 activity are often detected in cancers and constitutively active STAT3 is capable of transforming *in vitro* cultured cell lines (reviewed in (Calo et al., 2003)). STAT3 is implicated in the regulation of cell cycle and cell survival, likely through transcriptional targets such as *cMyc, CyclinD1* and *Bcl-XL* (Calo et al., 2003). LIF can also activate the PI3K pathway, a well known pro-survival signal and this may be significant if LIF further enhances PI3K activity in insulin-containing media where constitutive PKB phosphorylation is already observed (see chapter 3). Similarly, GSK3 can negatively regulate many cell processes implicated in cell metabolism and is involved in cell cycle regulation. GSK3 phosphorylates targets including cMyc (He et al., 1998; Gregory et al., 2003) and CyclinD1 (Diehl et al., 1998), targeting them for proteolysis and negatively regulates protein synthesis through phosphorylation of targets such as eIF2 (eukaryotic translation initiation factor 2) (Welsh et al., 1998). It is likely that the modulation of pathways such as these is an important aspect of the promotion of self-renewal by LIF and GSK3 inhibition.

The question of enhanced cell survival was addressed through the forced expression of Bcl2. Bcl2-expressing ES cells cultured in higher concentrations of PD03 exhibited reduced differentiation as compared to cultures in PS but morphologically differentiated cells were never totally eliminated from the culture despite ERK phosphorylation being undetectable by immunoblotting. This shows that the combination of inhibition of differentiation by blocking ERK signalling and inhibition of apoptosis through forced expression of Bcl2 does not reproduce the efficient self-renewal observed in 3i or in standard ES cell culture conditions. Therefore, both LIF and CHIR must have effects that extend beyond the inhibition of apoptosis.

4.3.3 ERK Signalling is dispensable for self-renewal of ES cells

ES cells are somewhat unique in their apparent independence from the ERK signalling pathway. Robust culture in high concentrations of the MEK-inhibitor,

PD03, demonstrates that the cells can survive and proliferate when ERK activity is low or absent. In somatic cells transit through the G1/S checkpoint very often requires active ERK signalling and CyclinD1 expression is under the control of TFs activated by ERK (Treinies et al., 1999). In ES cells, however, CyclinD expression levels are low and do not appear to respond to activation of the ERK pathway (Jirmanova et al., 2002). Constitutive inactivation of retinoblastoma (RB) and constitutive expression of CyclinE/CDK2 are thought to promote entry of ES cells to S-phase (Stead et al., 2002). In fact, ES cells cannot be induced to arrest at G1/S (Schratt et al., 2001). A consequence of this is that somatic cells that appear in culture as a result of spontaneous differentiation cannot survive and are selectively eliminated. This may in part underlie our ability to culture ES cells through multiple passages in PS alone but little cell death is evident in 3i cultures and no overt differentiation is detected, even when Bcl2 is constitutively expressed, suggesting that the rate of spontaneous differentiation in 3i is low and that selection plays little or no part in long-term self-renewal in 3i. However, the anticipated bias for ES cell survival in conditions where ERK signalling is blocked provides us with a culture condition that will expand ES cells at the expense of somatic cells. This may have applications in reprogramming where pluripotent cells arising at a low frequency from cultures of somatic cells (Takahashi and Yamanaka, 2006) must be isolated and expanded (see general discussion).

4.3.4 How does ERK activity promote differentiation?

ERK activity downstream of FGF signalling promotes differentiation of ES cells (Burdon et al., 1999b; Kunath et al., 2007; Stavridis et al., 2007) and compromised signalling in this pathway prevents primitive endoderm differentiation in the blastocyst (Chazaud et al., 2006). However, the downstream targets involved in promoting differentiation are not yet known. Perhaps the most informative piece of evidence is that inhibition of differentiation by blocking the FGF4-MAPK signalling pathway is not lineage-restricted (Kunath et al., 2007). It therefore seems unlikely that ERK directly activates genes associated with commitment to a particular lineage. Rather, ERK signalling may function in ES cells to promote a state of responsiveness

to differentiation cues (Fig4.6). Furthermore, ERK activation does not result in inevitable differentiation since ERK activity is high in ES cells cultured in standard conditions. Under these circumstances differentiation appears to be inhibited by lineage-specific blockade of differentiation mediated by signals downstream of LIF and serum/BMP4 (Ying et al., 2003a). Inhibition of signalling downstream of FGF4 may remove the need to impose lineage-specific blockade of differentiation by maintaining ES cells in a state where they are unresponsive to signals promoting differentiation.

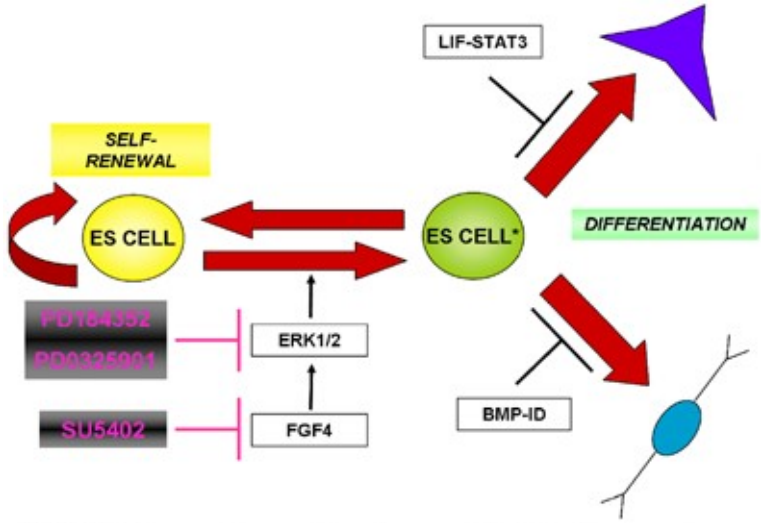


Fig4.6. Self-renewal proceeds autonomously if signals promoting differentiation are blocked. ES cells produce FGF4 which activates ERK1/2 and promotes the transition of ES cells to an unstable state where they are sensitive to signals promoting lineage specification. Differentiation along non-neural lineages is inhibited by LIF-STAT3 signalling while BMP induction of Id genes prevents neural differentiation. Pharmacological inhibition of the FGF4-ERK1/2 signalling pathway prevents cells from reaching a state sensitive to differentiation.

One hypothesis was that ERK signalling promotes down-regulation of Nanog. If this were the case Nanog expression would be expected to increase in response to PS or PD03. Indeed, ERK inhibition does result in elevated levels of Nanog protein and *Nanog* transcript levels are elevated in 3i relative to LIF and BMP4 culture conditions. However, while the elevated Nanog levels are likely to increase the efficiency of self-renewal (Chambers et al., 2007) the fact that *Nanog*-/- ES cells can be maintained in 3i demonstrates that elevated Nanog expression is not required for

the self-renewal phenotype observed in 3i. A direct comparison of cultures of *Nanog*-/- ES cells in N2B27 alone or plus PD03 clearly shows that differentiation is inhibited by the presence of the MEK inhibitor despite the absence of Nanog expression.

It has been suggested that in transcriptionally permissive ES cells expression of lineage-associated genes fluctuates at levels below a threshold required to induce differentiation until exposure to an environmental cue leads to initiation of a somatic transcriptional program and differentiation (Silva and Smith, 2008). Might ERK signalling serve to promote activation or stabilisation of somatic transcriptional programmes? At present this is not known but we can speculate on the predictions of such a hypothesis. Comparison of gene expression in self-renewal conditions with either high (LIF plus serum/BMP4) or low ERK activity (3i/2i) should reveal whether or not lineage-associated gene expression correlates with levels of ERK activity. Global transcriptional profiling or real-time PCR examination of a more limited set of candidate genes, selected to reflect markers of early differentiation, would be appropriate to examine this. For gene expression analysis on a population of ES cells it would be important to ensure that differentiated cells in the culture were not included. This could be achieved by using transgenic ES cells lines that express antibiotic resistance genes under the control of pluripotency-specific promoters or by sorting undifferentiated cells from the population using flow cytometry.

Active ERKs can regulate transcription through direct phosphorylation of transcription factors (Marais et al., 1993) and through direct or indirect modulation of nucleosome phosphorylation and acetylation (reviewed in (Hazzalin and Mahadevan, 2002)). The immediate early genes (IEGs), which include *cFos*, *Egr1* and *JunB*, are downstream targets that respond rapidly to ERK-mediated activation of TFs such as cFos (reviewed in (Whitmarsh, 2007)). ES cells lacking serum response factor (SRF) are unable to activate IEGs in response to mitogens but show no apparent compromise to their proliferation rate or ability to self-renew (Schratt et al., 2001). Interestingly, their colony-forming efficiency was lower than

heterozygous controls implying that there may be a role for these transcriptional targets in ES cells, although the authors suggest the difference may be caused by decreased plating efficiency as a result of down-regulation of actin expression. *Srf*-null ES cells were also found to differentiate inefficiently upon LIF withdrawal and in response to DMSO treatment (Weinhold et al., 2000). The authors of that work attributed the phenotype to a non cell-autonomous loss of mesodermal induction but did not rigorously test the ability of *Srf*-null ES cells to differentiate into other cell types. An examination of these cells in defined, serum-free conditions might reveal a more general compromise in their ability to differentiate. If the gene products activated by ERK signalling, in particular the IEGs, promote differentiation *Srf*-null ES cells would not differentiate efficiently. Similarly to *Fgf4*-null ES cells (see chapter 3), they may no longer require inhibition of the FGF4-MAPK signalling cascade for efficient self-renewal and may phenocopy 3i culture in the presence of CHIR alone. Furthermore, if ERK transcriptional targets have a role in ES cell viability and proliferation the *Srf*-null ES cells would be predicted to exhibit decreased colony forming efficiency, an effect that might be more readily observed in serum-free culture.

In addition to regulation of TF activity, MAPK signalling can regulate chromatin conformation. For example, phosphorylation of the co-activators CBP and p300 by ERK stimulates their histone acetyl transferase (HAT) activity (Liu et al., 1999; Gusterson et al., 2002). The resulting increase in histone acetylation is associated with increased transcription. In some cases nucleosome acetylation activity is required for efficient gene activation by MAPK signalling (Alberts et al., 1998). Furthermore, downstream targets of ERK, the mitogen- and stress-activated kinases-1 and -2 (MSK1/2), are known to phosphorylate histone H3 on residues Ser10 and Ser28 (Soloaga et al., 2003). Chromatin immunoprecipitation experiments show that H3-phosphorylation is associated with active IEGs. Together with the observation that Ras-transformed cells have increased H3 phosphorylation and a more open chromatin formation (reviewed in (Dunn et al., 2005)) this suggests that increased ERK activity may promote open chromatin and increased gene expression. If levels of lineage-associated gene transcription increase in response to ERK activity

this could increase the chances of initiating differentiation (Silva and Smith, 2008). It would be interesting to examine the activation status of MSK1/2 and the chromatin structure of ES cells in 3i culture conditions. If MSK1/2 are involved in promoting differentiation inhibition of their activity would be expected to recapitulate the effect of inhibiting ERK activation.

The work described in this chapter builds on a growing body of information implicating the ERK pathway in the promotion of differentiation. The fact that the phenotypes described for ES cells *in vitro* (Burdon et al., 1999b; Kunath et al., 2007; Stavridis et al., 2007) can be directly related to the failure of the ICM to form the primitive endoderm when this pathway is inactivated (Chazaud et al., 2006) gives us confidence that the phenomenon is of relevance to the behaviour of ES cell equivalents *in vivo*. This is not the case for other pathways implicated in the maintenance of ES cells with the exception of the role of signalling through the gp130 receptor in diapause (Nichols et al., 2001). It is hoped that these findings will translate to humans where the regulation of early differentiation events may be conserved and that this will allow us to establish 'true' ES cells from human embryos as opposed to existing lines which correspond more closely to the post-implantation epiblast and EpiSC lines (Brons et al., 2007; Lovell-Badge, 2007; Tesar et al., 2007).

Chapter 5: The role of GSK3 inhibition in the culture of mouse embryonic stem cells

5.1 Introduction

CHIRON99021 (Nikoulina et al., 2002) (CHIR) is a critical component of 3i. Its removal from 3i to leave only PS results in a degree of spontaneous differentiation into neurons and a severe compromise in cell viability and proliferation (see chapter 4). In this chapter the role of this small molecule inhibitor in self-renewal is examined by investigating candidate targets that are predicted to be affected by the inhibition of GSK3.

5.1.1 CHIRON99021

The choice of CHIR in the wider context of GSK3 inhibition has already been discussed (see chapter 3) but here the inhibitor is described in more depth. A series of substituted aminopyrimidines were generated and found to be capable of inhibiting GSK3 activity at concentrations in the nanomolar range (Nikoulina et al., 2002). Testing of two such compounds, named CHIR98014 and CHIR99021, revealed that they inhibited the activity of GSK3-α and –β at concentrations at least 500 times lower than the effective concentrations obtained for a panel of 20 other kinases which included the closest homologues of GSK3, Cdc2 and Erk2 (Nikoulina et al., 2002). They act as inhibitors of ATP binding and were similarly potent against both isoforms of GSK3.

GSK3 is a promising target for the treatment of TypeII diabetes. In resting cells GSK3 is active and phosphorylates and inactivates its substrate glycogen synthase (GS) (Embi et al., 1980). Insulin secretion in response to elevated blood sugar results in activation of PI3K/PKB signalling, phosphorylation and inhibition of GSK3 and subsequent release of GS from inhibition (Alessi and Cohen, 1998). TypeII diabetics are insulin-insensitive and this pathway operates less effectively leaving them prone

to episodes of hyperglycemia. It is hoped therefore that direct inhibition of GSK3 might restore GS activity and glycogen synthesis in patients who do not respond to insulin. Promisingly, both CHIR compounds were shown to increase GS activity and to reduce hyperglycemia in Rat diabetes models (Cline et al., 2002; Nikoulina et al., 2002). More recently, CHIR99021 has been tested against a larger panel of kinases and compared directly to other well known GSK3 inhibitors. It was found to be both the most potent and the most specific inhibitor and was recommended by the authors for use in the study of GSK3 (Bain et al., 2007).

5.1.2 Upstream and downstream of GSK3

GSK3 is regulated by multiple signalling pathways and phosphorylates many targets, usually resulting in their inactivation (reviewed in (Doble and Woodgett, 2003)). It exists in a constitutively active state and is negatively regulated in response to various signals. Phosphorylation on an N-terminal serine (Ser21 for GSK3-α and Ser9 for GSK3-β) inhibits the kinase activity of the enzyme (McManus et al., 2005). The phosphorylated serine acts as a "pseudosubstrate" for the active site of the enzyme, preventing it from binding to other phosphorylated residues. Ser9/21 phosphorylation can be catalysed by several kinases including S6K, RSK, PKA, PKC and the best known, PKB (Cross et al., 1995). This makes GSK3 sensitive to regulation by growth factors and changes in nutrient availability. The best studied example is phosphorylation by PKB in response to insulin signalling, allowing insulin to increase glycogen synthesis by de-inhibition of GS (Fig5.1) (McManus et al., 2005). This mechanism of inhibition reflects GSK3's preference for primed substrates. GSK3 recognises a motif of Ser/ThrXXXSer/Thr-P (where X is any amino acid and P indicates phosphorylation). The N-terminal Ser/Thr is the residue targeted for phosphorylation and the C-terminal Ser/Thr is phosphorylated, serving as a priming site for recruitment of GSK3. GSK3 is thought to bind to the phosphorylated residue, positioning its kinase domain optimally relative to the target site (reviewed in (Doble and Woodgett, 2003)).

There are other GSK3 targets that are regulated in a similar manner to GS. That is, they are negatively regulated and released from inhibition following Ser9/21 phosphorylation of GSK3. The activity of TF c-Jun is inhibited by GSK3 phosphorylation while c-Myc is targeted for proteolysis. CyclinD1 is also targeted for proteolysis (Diehl et al., 1998), implicating GSK3 in the regulation of cell cycle while inhibition of eukaryotic translation initiation factor 2B (eIF2B) reduces protein synthesis by inhibiting translation (Welsh et al., 1998). GSK3 activity can be implicated in activities as diverse as glycogen metabolism, translation and apoptosis all of which are therefore subject to regulation by pathways promoting phosphorylation of GSK3 on Ser9/21.

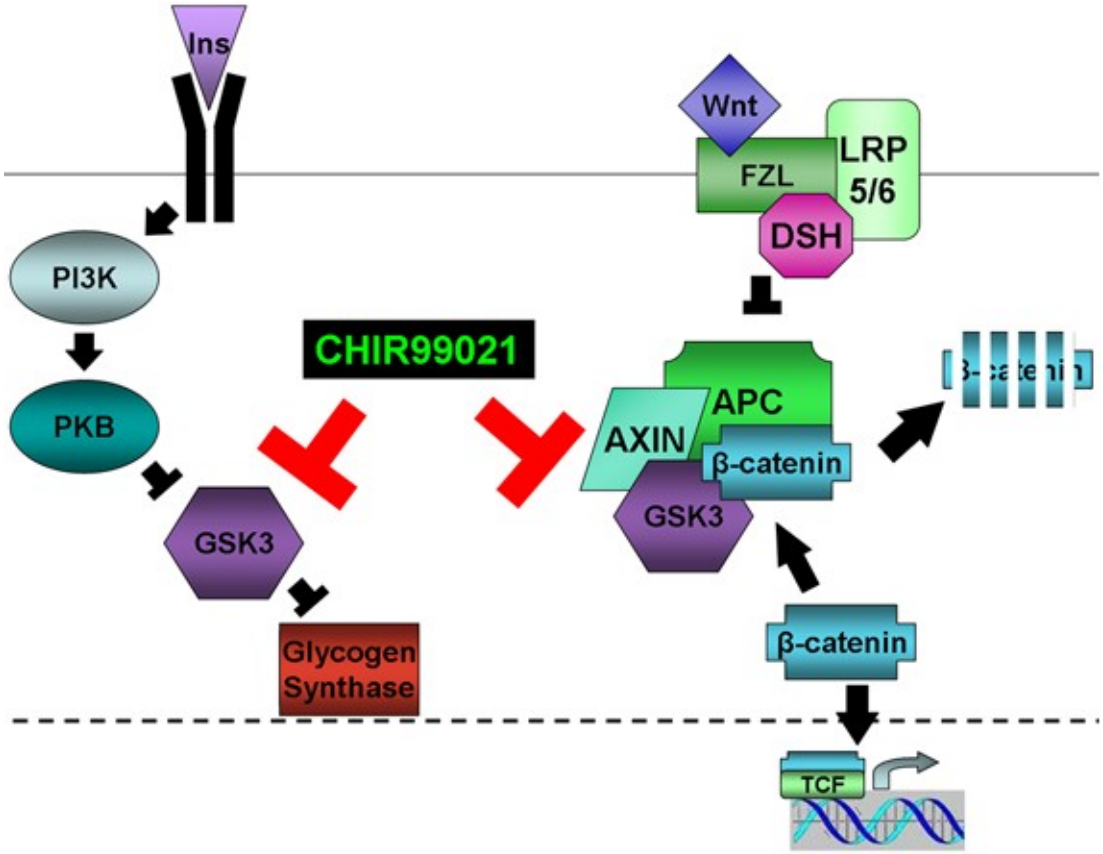


Fig5.1. Schematic of GSK3 in insulin and canonical Wnt signalling. Left: Insulin binds and activates its receptor. Downstream signalling through PI3K and PKB phosphorylates and inactivates GSK3, releasing glycogen synthase from inhibition. Many GSK3 targets are known or predicted to be regulated in a similar manner. Right: Cytoplasmic β-catenin is targeted for proteolysis by the destruction complex. Binding of Wnt ligands to the receptor Frizzled (FZL) results in recruitment of low-density lipoprotein (LDL)-receptor-related protein 5 or 6 (LRP5/6) and dishevelled (DSH). Signalling downstream inhibits the activity of the destruction complex and allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus. Nuclear β-catenin associates with DNA-bound TCF/LEF co-factors and activates transcription of target genes. Inhibition of GSK3 by CHIR reduces GSK3-mediated phosphorylation of glycogen synthase and β-catenin, simultaneously mimicking the effects of insulin and Wnt signalling.

GSK3 is also regulated by Wnt signalling (Fig5.1). GSK3, together with proteins including Axin, APC and β-catenin, forms a complex known as the destruction complex. This complex is active in 'resting' cells, phosphorylating β-catenin and targeting it for ubiquitin-dependent proteolysis (reviewed in (Clevers, 2006)). GSK3 is the kinase responsible for β-catenin phosphorylation. β-catenin is 'primed' by casein kinase I (CKI)-mediated phosphorylation on Ser45 and subsequently phosphorylated by GSK3 on residues Thr41, Ser37 and Ser33 (reviewed in (Patel et al., 2004)). GSK3 is also thought to modulate the activity of the destruction complex by phosphorylating its components. Phosphorylation of Axin, which is believed to serve as a scaffolding protein for the complex, results in its stabilization and increased affinity for β-catenin (Rubinfeld et al., 1996; Jho et al., 1999; Yamamoto et al., 1999) while phosphorylation of APC also increases its binding to β-catenin (Rubinfeld et al., 1996). Active GSK3 therefore regulates β-catenin proteolysis by promoting its interaction with the destruction complex and by directly phosphorylating it.

When exposed to Wnt ligands β-catenin phosphorylation is reduced and the protein is stabilized. Wnt ligands bind to the transmembrane receptor, Frizzled (Fz), which can cooperate with another receptor, low-density lipoprotein (LDL)-receptor-related protein 5 or 6 (LRP5/6). LRP5/6 is required for transmission of the Wnt signal. Surprisingly, activation of LRP5/6 and transmission of the Wnt signal requires phosphorylation of LRP5/6 by GSK3 and Casein Kinase-Iγ (CKIγ) (Davidson et al., 2005; Zeng et al., 2005). Phosphorylation is activated through a poorly defined mechanism in response to Wnt ligands and appears to occur sequentially, with GSK3-mediated phosphorylation first (Zeng et al., 2005). In this instance GSK3 appears to act in the absence of priming phosphorylation. The activity of both GSK3 and CKIγ is required for the activation of a TCF/LEF reporter (Zeng et al., 2005).

The most up to date model of Wnt signalling proposes that ligand binding to Fz results in recruitment of LRP5/6 and Dishevelled (Dsh), another protein crucial for transmission of the Wnt signal. Dsh in turn recruits Axin to which GSK3 is known to bind. In this manner it is thought GSK3 is brought into contact with LRP5/6 where it

can mediate phosphorylation of motifs on the LRP5/6 intracellular domain, creating binding sites for the recruitment of Axin to LRP5/6 which it is known to bind (Zeng et al., 2008). Axin-bound GSK3 may then further phosphorylate LRP5/6, fully activating the receptor.

The conflicting effects of GSK3 in this signalling pathway, both positive and negative regulation, appear to be mediated by membrane-bound and cytoplasamic pools respectively. Expression of wild-type GSK3 antagonizes Wnt3a activation of the TCF/LEF reporter whereas expression of a membrane-bound form of GSK3 synergizes with Wnt3a to promote TCF/LEF reporter activity (Zeng et al., 2005). In a complementary experiment expression of a peptide capable of binding and inhibiting GSK3 activated Wnt signalling but expression of the same protein fused to a motif that directs it to the membrane actually inhibits signalling (Zeng et al., 2008). Thus, GSK3 is involved in the regulation of canonical Wnt signalling at multiple levels and precisely how binding of Wnt ligands to Fz results in a decrease of GSK3-mediated β-catenin phosphorylation remains unclear.

Axin is a limiting factor in Wnt signalling. Increasing its levels decreases β-catenin activity and can rescue the loss of destruction complex function observed upon loss of APC (reviewed in (Clevers, 2006)). Loss of Axin results in a severe reduction of Wnt-induced LRP5/6 phosphorylation (Zeng et al., 2008), suggesting that Axin is required for activation of Wnt signalling, but the absence of Axin from the destruction complex is epistatic to this, abolishing β-catenin phosphorylation and proteolysis. Similarly, the loss of GSK3 totally abolishes activation of LRP5/6 but its absence from the destruction complex releases β-catenin from inhibition (Doble et al., 2007). GSK3-inhibitors would be predicted to inhibit both positive and negative regulation of Wnt signalling but the outcome is determined by the most downstream effect, inhibition of GSK3 in the destruction complex.

There are likely to be multiple levels of control in β-catenin regulation but notably the amount of GSK3 does not become limiting until at least 3 of the 4 alleles encoding the two isoforms are knocked out (Doble et al., 2007). Thus, although

GSK3 is the enzyme ultimately responsible for targeting β-catenin for destruction, regulation of this activity is complex and may be mediated through modulation of structural components of the destruction complex.

Stabilized β-catenin accumulates in the cytoplasm in response to Wnt signalling before translocating to the nucleus. In the nucleus β-catenin displaces corepressors such as Groucho from DNA-bound TCF/LEF TFs, activating transcription of TCF/LEF target genes including components of the destruction complex, demonstrating another layer of autoregulation in the Wnt signalling pathway. Other target genes, such as *cMyc* and *CyclinD1*, have created a great deal of interest because of their implications for cancer. Mutations affecting the destruction complex or β-catenin itself are frequently found in cancers (reviewed in (Clevers, 2006)).

It is important to note that the two mechanisms described above for the regulation of GSK3 act independently. Insulin/PKB-mediated GSK3 inhibition does not result in stabilization of β-catenin while Wnt signalling does not lead to Ser9/21 phosphorylation of GSK3 or result in decreased phosphorylation of targets such as GS. Several elegant studies have demonstrated this. ES cells which carry knock-in mutations of Ser9/21 become insensitive to insulin signalling but retain their Wnt response (McManus et al., 2005). Similarly, ES cells lacking wild-type GSK3 in which GSK3α carrying a substitution of Ser21 has been expressed re-establish Wnt-sensitive control of β-catenin (Doble et al., 2007) clearly demonstrating the independence of this pathway from Ser21 phosphorylation. This separation of GSK3 activities is likely caused by the incorporation of a proportion of GSK3 into the destruction complex where it is inaccessible to the kinases that mediate Ser9/21 phosphorylation and is regulated exclusively by Wnt signalling (reviewed in (Clevers, 2006)). Thus, it is important to bear in mind that pharmacological inhibitors of GSK3 will simultaneously mimic the effects of canonical Wnt signalling and of inhibition by Ser9/21 phosphorylation, a situation that cannot be completely recapitulated by the activation of any one signalling pathway.

5.2 Results

5.2.1 ES cell culture in CHIR99021

In chapter three I provided evidence that the effect of CHIR is specific to GSK3 inhibition and that CHIR activates canonical Wnt signalling and leads to dephosphorylation of known GSK3 targets. However, the effects of CHIR on molecular targets tell us nothing in themselves about how CHIR regulates ES cell self-renewal. As a first step towards elucidating this I examined the morphology and growth of cells cultured with CHIR in combination with different factors known to modulate self-renewal.

Cells cultured in CHIR alone, using N2B27 as a basal medium, initially proliferated as morphologically undifferentiated cells in three-dimensional colonies similar to those observed in 3i (Fig5.2a). However, with increasing time in culture differentiated cells emerged (Fig5.2a) and increasing cell death was observed upon passaging. Cultures could be maintained with the presence of morphologically undifferentiated cells for several passages (Fig5.2.1) but eventually differentiation and cell death mean that the cells can no longer be passaged. The differentiated cells that emerged were non-neural (Fig5.2a,b), consistent with a role for Wnt signalling in the inhibition of neural differentiation (Aubert et al., 2002; Haegele et al., 2003), which would normally proceed in N2B27 alone (Ying et al., 2003b). The persistence of undifferentiated cells for at least 7 passages (Fig5.2.1) is consistent with a partial block of differentiation as cells cultured in N2B27 alone cannot be passaged more than once. The highly proliferative cells observed in the initial culture also suggest that CHIR maintains cell proliferation. Contrast this effect with PS cultures where differentiation was mostly blocked but proliferation was poor (Fig5.2a). The non-neural differentiation observed also suggested that CHIR promotes differentiation or alters the fate of spontaneously differentiating cells. I conclude that GSK3-inhibition has pleiotropic effects on ES cells and is not sufficient to support long-term culture.

BMP signalling has been demonstrated to promote self-renewal in the presence of LIF by blocking neural differentiation. As neural differentiation does not occur in the presence of CHIR it seemed unlikely that the addition of BMP4 would have a positive effect on self-renewal. However, it has been suggested that BMP4 can promote self-renewal by modulating MAPK signalling pathways (Qi et al., 2004) and the effect of any signalling pathway is altered by the context in which it is activated. When ES cells were cultured in the presence of BMP4 and CHIR there was little difference as compared to CHIR alone other than a morphological change in the differentiated cells (Fig5.2b). Interestingly, however, the normally potent differentiation-inducing activity of BMP4 appeared to be partially suppressed as judged by the persistence of ES cells over several passages (Fig5.2b), which is never seen in BMP4 alone (Ying et al., 2003a), and the absence of the large, flattened cells characteristic of BMP4-induced differentiation (Fig5.2b). The effect of BMP4 on culture in the presence of CHIR was further investigated by attempting to maintain cultures long-term. BMP4 addition to 3i slightly increased the amount of spontaneous differentiation observed but cultures could readily be maintained (Fig5.2.1). ES cells cultured in CHIR and BMP4 were highly heterogeneous but could be maintained for at least 7 passages and retained a population of cells that express Oct4 (Fig5.2.1), a phenotype similar to that seen for CHIR alone (Fig5.2.1). These results suggest that GSK3 inhibition not only inhibits neural differentiation but also prevents BMP-induced differentiation. It appears that GSK3 inhibition restricts differentiation to a limited set of lineages but does not homogeneously drive ES cells to differentiate. In fact, persisting ES cells expand and can be passaged, indicating that, at least in bulk culture, GSK3 inhibition helps maintain a population of ES cells.

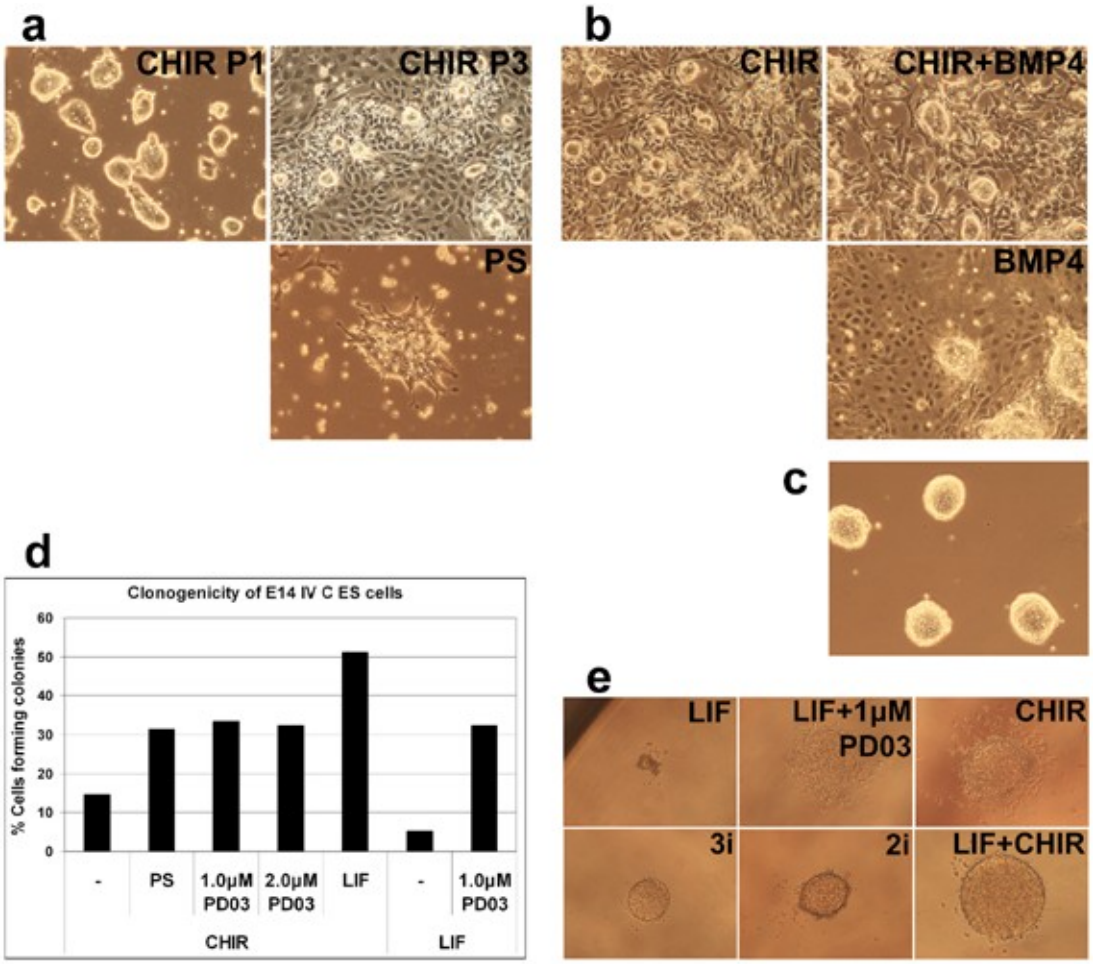


Fig5.2. ES cells exhibit a pleiotropic response to CHIR a) Phase contrast images of E14Tg2a ES cells after one (P1) or three (P3) passages in N2B27 plus CHIRON99021 (CHIR), 3μM alone or 2 passages in N2B27 plus PS. b) Phase contrast images of E14Tg2a ES cells after three passages in N2B27 plus CHIR, 3μM; CHIR, 3μM+BMP4; or BMP4 alone. c) Phase contrast images of E14Tg2a ES cells after two passages in N2B27 plus LIF+CHIR, 3μM. d) Histogram showing clonogenicity of E14 IV C ES cells. Single cells were plated in 96-well plates in N2B27 plus LIF or CHIR alone (-) or plus PS; PD0325901 (PD03), 1 or 2 μM; or LIF. ES colonies were counted after 10 days' culture. e) Representative phase contrast images of of colonies formed as described in (d).

LIF is the best characterised of the pro-self-renewal signalling molecules. It appears to prevent differentiation by inhibiting the differentiation-inducing effects of serum or BMP4 and is also likely to play a role in cell survival and proliferation. It was reasoned that the combination of LIF and CHIR might inhibit non-neural and neural differentiation respectively and in doing so promote self-renewal. In fact, it has already been reported that LIF and Wnt signalling can synergize to promote efficient self-renewal (Ogawa et al., 2006). ES cells cultured in a combination of LIF and CHIR formed highly refractile, rounded up colonies with an extremely regular undifferentiated morphology (Fig5.2c). They could be passaged for an extended

period in culture with little evidence of differentiation and formed colonies from single cells with efficiency as high as that seen for any other growth condition tested (Fig5.2d). The colonies formed in LIF plus CHIR also tended to be larger and more regular in appearance (Fig5.2e). It appears that this combination effectively blocks differentiation and the high clonogenicity suggests that cell survival is enhanced. A similar effect on clonogenicity and colony size was observed when LIF was added to 3i (see chapter 3) providing further evidence that LIF and CHIR have additive effects on cell survival and/or proliferation. Similarly, cells lacking GSK3 (DKO cells) exhibited significantly enhanced self-renewal in the presence of LIF although LIF is insufficient to block the spontaneous differentiation observed in DKO cells (see chapter 3). This implies a dosage effect with optimal self-renewal occurring upon incomplete GSK3 inhibition while a total lack of GSK3 promotes differentiation that cannot be blocked by the addition of LIF, although it is effectively blocked by inhibition of ERK activity.

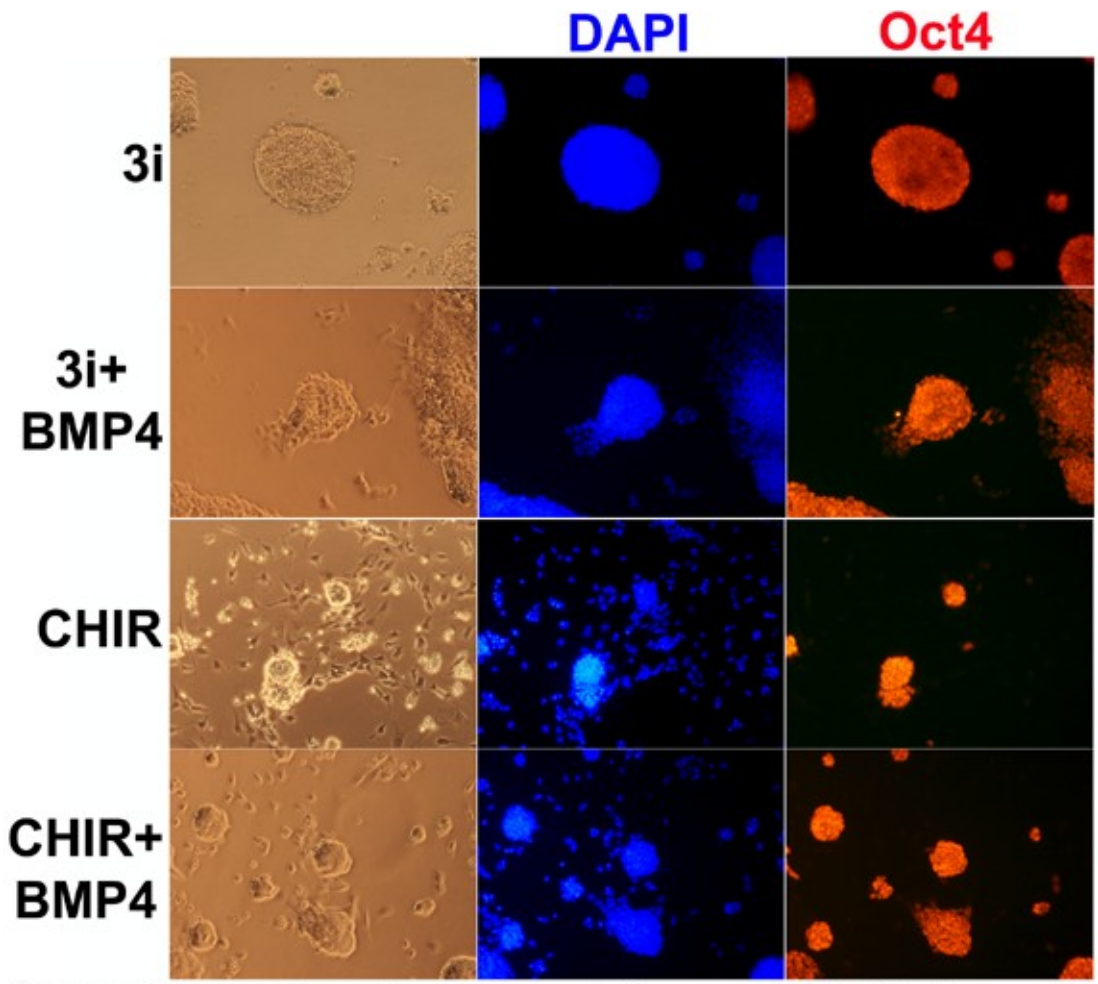


Fig5.2.1. BMP4 does not enhance ES cell self-renewal in the presence of CHIR. After 7 passages in N2B27 plus 3i; 3i+BMP4; CHIRON99021 (CHIR), 3μM; or CHIR+BMP4 E14Tg2a ES cells were fixed and immunostained for Oct4 expression.

To further investigate the interaction between LIF and GSK3 inhibition a chimeric receptor that permits activation of STAT3 by the addition of the cytokine GCSF but does not activate ERK1/2 (Burdon et al., 1999b) was utilised. The intracellular domain of gp130 is fused to the extracellular domain of the GCSF receptor and addition of GCSF causes receptor dimerization and activation. The tyrosine residue (Y118) responsible for binding Shp2 has been substituted for phenylalanine (Y118F) to prevent activation of ERK1/2. This mutation has the added effect of preventing SOCS3-mediated negative feedback and promotes hyperactivation of STAT3 at higher concentrations of GCSF. Lines stably expressing the chimeric receptor were generated by electroporating E14Tg2a ES cells with linearised plasmid DNA encoding the fusion protein and selecting for zeocin resistance. Colonies were picked after 10-12 days of selection and expanded to form clonal lines termed ‘Y118F’.

Clones were readily established and were indistinguishable from parental cells when cultured in standard conditions.

The receptor can be activated by the addition of media conditioned with GCSF (GCSF-CM). Conditioned media is generated by transiently transfecting Cos7 cells with a plasmid encoding GCSF and harvesting the media in which they are grown. The activity of GCSF-CM was tested by treating a previously described cell line (Burdon et al., 1999b), generated by introducing the chimeric receptor into D027 ES cells which express β-galactosidase from the Oct4 locus, with increasing concentrations of GCSF-CM and assessing the expression of β-galactosidase in cell lysates. As previously described, total β-galactosidase activity initially increased with increasing GCSF but was reduced at high concentrations (Fig5.3a). This GCSF-CM was subsequently used to assess the phenotype of Y118F ES cells.

The activation of STAT3 was assessed in the clonal line, Y118F(1), in response to increasing concentrations of GCSF-conditioned media. A STAT3-responsive luciferase reporter was activated in a dose-dependent manner (Fig5.3b). Even dilutions of the conditioned media of 1:20000 produced an effect on STAT3 activation comparable to that seen with saturating concentrations of LIF. The low amounts of conditioned media used at these dilutions argue against the possibility of unintended effects of the conditioned media. The effect of combining GCSF with 3μM CHIR was assessed at a range of concentrations and it was found that together they promoted self-renewal more efficiently than either did separately. At low concentrations of GCSF, which did not support efficient self-renewal (1:80000 dilution), the addition of CHIR fully restored the formation of undifferentiated colonies at clonal density (Fig5.3c). I conclude that STAT3 activation and GSK3 inhibition act together to promote efficient self-renewal. Specifically, GSK3 inhibition lowered the threshold for STAT3 activation required for optimal self-renewal. This is in agreement with Ogawa *et al* who found that Wnt3a supported ES cell self-renewal at sub-threshold concentrations of LIF (Ogawa et al., 2006). This may in part explain the behaviour of ES cells cultured in CHIR alone if there is a degree of paracrine activation of STAT3 signalling in bulk cultures.

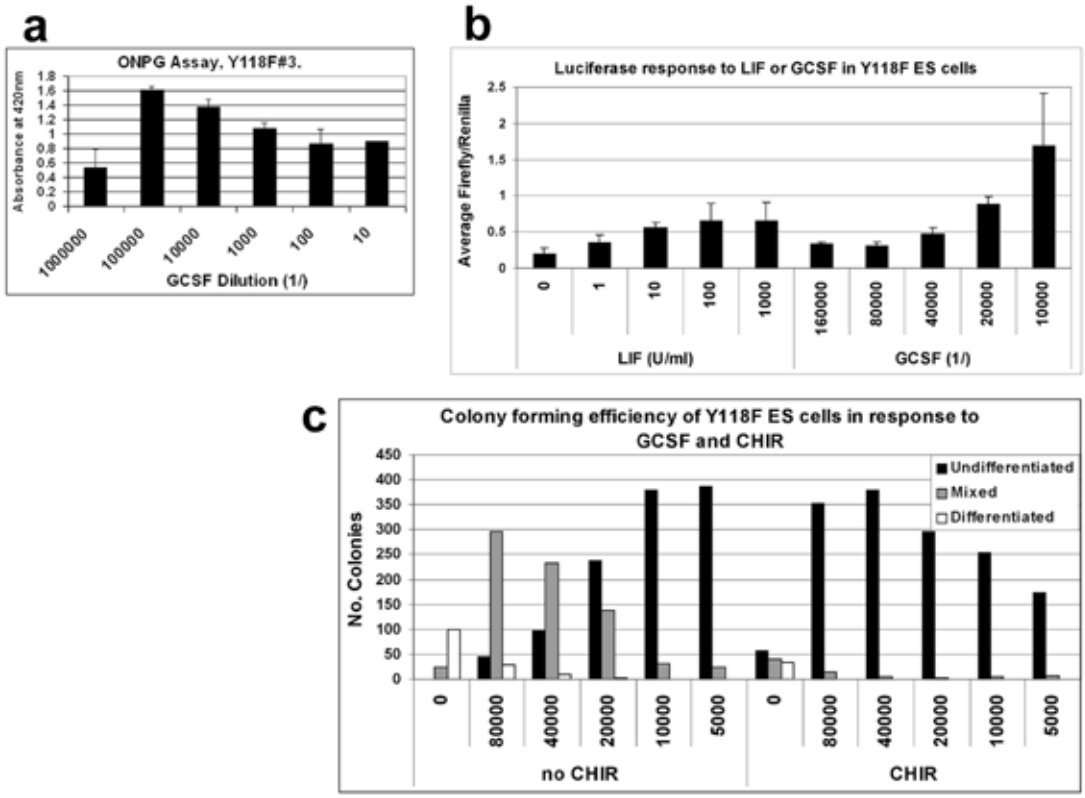


Fig5.3. GSK3 inhibition promotes STAT3 driven self-renewal. a) ONPG Assay. Histogram showing absorbance at 420nm representing β-galactosidase expression from the Oct4 locus in ES cell lysates after 6 days' culture in the presence of GCSF-conditioned media (CM) at the indicated dilutions. Error bars represent standard deviation of the mean for two biological replicates. b) Histogram showing activity of the STAT3-responsive APRE luciferase reporter in ES cells stimulated with LIF or GCSF-CM at the indicated concentrations. Error bars represent standard deviation of the mean for three biological replicates. c) Histogram showing numbers of alkaline phosphatase (AP) positive (undifferentiated), negative (differentiated) and mixed colonies formed from 600 cells after 6 days' culture in the presence or absence of CHIR plus GCSF-CM at the indicated concentrations.

From our initial observations of 3i ES cell culture it is clear that blockade of the ERK1/2 signal eliminates the spontaneous differentiation seen in CHIR99021 cultures and allows self-renewal to proceed efficiently (see chapter 3). By examining the effects of combining CHIR with two other self-renewal factors, LIF and BMP4, I have established that LIF but not BMP4 can promote self-renewal in this context. The evidence suggests that CHIR promotes self-renewal only when its differentiation promoting activities are blocked and that this can be achieved by imposing a general restriction on differentiation through ERK inhibition (Kunath et al., 2007) or by blocking non-neural differentiation through activation of STAT3 (Niwa et al., 1998; Ying et al., 2003a). Furthermore, the effects of LIF and CHIR on cell viability, which

are clearly observed upon the addition of either to PS, can be combined to further enhance viability, as evidenced by increased clonogenicity (Fig5.1d). This implies that LIF and CHIR activate independent pathways capable of functioning in parallel to enhance self-renewal. The targets of these pathways remain to be identified. They may be distinct or the pathways may converge on a common effector(s).

5.2.2 A role for Canonical Wnt Signalling?

As described above, GSK3 inhibition can mimic the effects of canonical Wnt signalling by preventing β-catenin phosphorylation on the residues that target it for proteolysis (Clevers, 2006). Several reports have attributed the effects of GSK3 inhibitors to activation of β-catenin and have shown that similar phenotypes are observed upon addition of Wnt ligands (Sato et al., 2004; Ogawa et al., 2006; Takao et al., 2007). However, these studies are conflicting in their conclusions and often rely on the use of relatively unspecific GSK3 inhibitors and conditioned media as a source of Wnt ligands. I wished to investigate the significance of β-catenin activation for the phenotype observed with CHIR.

CHIR reduces β-catenin phosphorylation and activates a TCF/LEF reporter in a dose-dependent manner (see chapter 3). TCF/LEF reporter activation was assessed in response to recombinant Wnt3a and compared directly to the effect of CHIR. Wnt3a elicited a dose dependent response from the TCF/LEF reporter but to obtain an equivalent level of activation to that observed with 3μM CHIR high concentrations (~100ng/ml) of Wnt3a were required (Fig5.4a). To assess the effect of Wnt3a on ES cells Wnt3a was substituted for CHIR in 3i at a range of concentrations. It was capable of supporting a limited degree of self-renewal at concentrations approaching 100ng/ml. The cells assumed a morphology similar to that observed in 3i, ie. rounded up colonies (Fig5.4b). However, spontaneous differentiation was more evident, the cells proliferated relatively poorly in bulk culture and the robust self-renewal observed in 3i was never reproduced. To further test this clonal assays were performed and the colony forming efficiency of ES cells in PS, 3i or PS plus Wnt3a scored (Fig5.4c). Virtually no colonies formed in PS alone and those that did were

either extremely small or extensively differentiated (Fig5.4d). Addition of Wnt3a increased the number of colonies formed (Fig5.4c) and the appearance of the colonies formed was shared between completely undifferentiated and colonies surrounded by a skirt of differentiation (Fig5.4d). However, Wnt3a failed to fully recapitulate the results obtained in 3i where more colonies were formed, a higher proportion of which were totally undifferentiated (Fig5.4c,d). The effect of Wnt3a may be to inhibit the residual neural differentiation observed in PS as this was eliminated in PS plus Wnt3a cultures but additional effects cannot be ruled out.

The best characterised signal downstream of Wnt ligands is activation of β-catenin. Activation of β-catenin independent of Wnt signalling can be achieved by expressing a stabilized form of β-catenin although high levels of transgene expression elicit a hyperactivation phenotype. Stabilized β-catenin lacks the residues normally phosphorylated by GSK3 and is therefore immune to proteolysis. A fusion of stabilized β-catenin and a modified oestrogen receptor (β-cateninER), which is activated by the addition of 4-hydroxy tamoxifen (4OHT), was used. ES cells stably expressing this fusion protein were generated (transfection performed by Q-L. Ying) and used to assess the effect of β-catenin activation on self-renewal in the context of PS (Fig5.4e). In PS alone both parental and β-cateninER cells grew poorly with a high degree of cell death. This suggests that expression of the construct does not confer a strong phenotype in the absence of 4OHT. Both lines exhibited rounded up morphology in CHIR alone and a degree of spontaneous differentiation although the β-cateninER appeared to have a higher proportion of morphologically undifferentiated cells suggesting clonal differences or a 4OHT-independent effect of β-cateninER expression. As expected, addition of 4OHT resulted in morphological changes similar to those observed with Wnt3a in the β-cateninER but not the parental cell line, indicating that the 4OHT had no effect on wild-type cells. Similarly to Wnt3a, activation of β-catenin in the presence of PS enhanced self-renewal as compared to PS alone. Rounded up, morphologically undifferentiated colonies formed but the degree of cell death was higher than that observed in 3i. With continued passaging β-cateninER cells cultured in PS+4OHT deteriorated. These results indicate that while stabilized β-catenin expression causes morphological

changes similar to those caused by CHIR and enhances ES cell growth relative to that observed in PS alone it cannot fully replace CHIR.

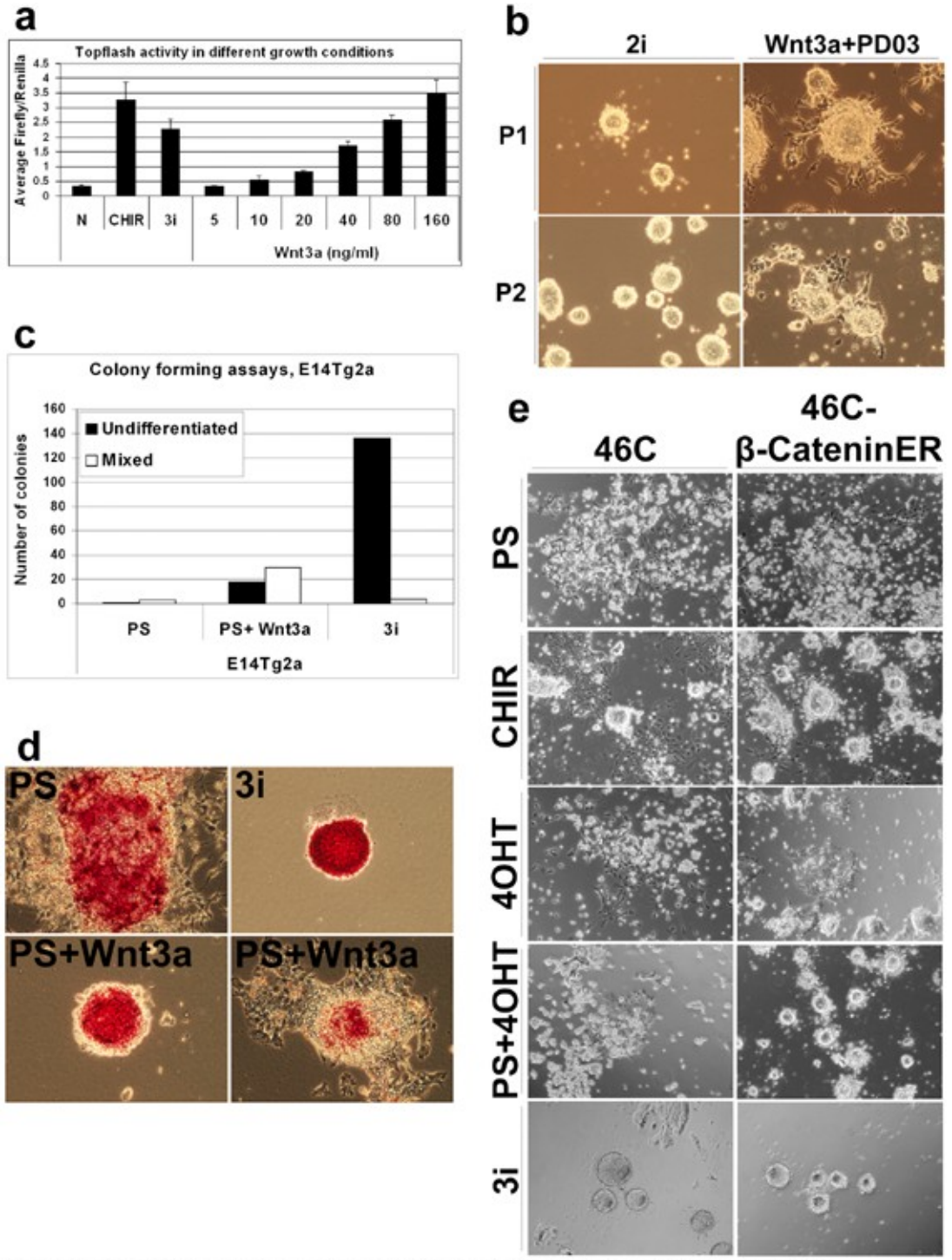


Fig5.4. Canonical Wnt signalling only partially reproduces the effect of GSK3-inhibition.
a) Histogram showing TOPFlash luciferase activity in E14Tg2a ES cells cultured in N2B27 alone (N) or plus CHIRON99021 (CHIR), 3μM; 3i; or Wnt3a at the indicated concentrations. Error bars represent standard deviation of the mean for 3 biological replicates. b) Phase contrast images of E14Tg2a ES cells cultured in CHIR+PD0325901 (PD03), 1μM (2i) or 100ng/ml Wnt3a+PD03 for 1 (P1) or 2 (P2) passages. c) Histogram showing number of colonies formed by E14Tg2a ES cells from 600 cells plated in N2B27 plus PS; PS+Wnt3a, 100ng/ml; or 3i. d) Images of alkaline phosphatase-stained colonies from (c). e) Phase contrast images of parental 46C ES cells or 46C ES cells stably expressing β-cateninER cultured for 2 passages in N2B27 plus PS; CHIR, 3μM; 4-hydroxy tamoxifen (4OHT), 100nM; PS+4OHT, 100nM; or 3i.

From these experiments I conclude that β-catenin activation may in part mediate the effect of CHIR in 3i, perhaps by promoting the expression of TCF/LEF target genes that antagonise neural differentiation. The morphology of the ES cell colonies suggests that β-catenin stabilisation is responsible for the rounded up morphology of cells cultured with CHIR. This may be caused by increasing the amount of β-catenin available for incorporation into adherens junctions and an increase in cell-cell interactions. It remains unclear from these findings, however, what additional effects CHIR has on self-renewal and whether β-catenin stabilisation is indispensable for efficient self-renewal in 3i.

One effect of β-catenin stabilisation is the activation of TCF/LEF target genes. Robust activation of the TCF/LEF reporter, TOPFlash (Fig5.4a), and upregulation of known TCF/LEF target genes *Brachyury, Axin* and *Cdx1* in response to CHIR was observed (Fig5.9c). This transcriptional activation can be inhibited by the expression of a dominant negative form of the human TCF/LEF TF, hLef1, termed ΔN-hLef1. This protein lacks the N-terminal domain responsible for interaction with β-catenin and is not therefore activated upon accumulation of β-catenin in the nucleus but acts as a constitutive repressor of its target genes. ES cells stably expressing ΔN-hLef1 were generated (Q-L. Ying). The cells were readily expanded and grew indistinguishably from the parental cell line. The TCF/LEF reporter response in these cells was assessed and a small but reproducible reduction in the response to CHIR observed. As the block to TCF/LEF transcription was incomplete it was not possible to assess the possibility that it is completely dispensible for self-renewal in 3i. However, a competition assay was performed to assess whether down-regulated TCF/LEF transcription has any effect on self-renewal (Ying *et al*, in press). The ΔN-hLef1 transgene could be excised from the genome by expression of Cre, activating GFP expression from a cassette downstream. Cre-excision was performed and GFP positive and negative cells mixed at a ratio of 1:1 in either serum plus LIF or in 3i. The proportion of GFP positive cells was examined after several passages. In 3i the proportion of GFP+ cells decreased implying that the expression of ΔN-hLef1 (in the GFP- cells) actually conferred a growth advantage. In serum and LIF conditions the

ratio of GFP+ to GFP- cells did not change. These results suggest that TCF/LEF signalling does not promote self-renewal in 3i and may in fact antagonize self-renewal.

Several attempts were made to further block TCF/LEF transcription by episomally expressing ΔN-hLef1. Episomal expression allows for highly elevated transcription (Chambers et al., 2003). However, ES cells could not be maintained in any condition when episomal expression was attempted. This suggests that a total block of TCF/LEF transcription is incompatible with ES cell propagation or that expression of the construct at these levels has non-specific toxic effects.

In a further attempt to address the role of β-catenin in 3i-directed self-renewal cells genetically deficient for *β-catenin* (Anton et al., 2007) were obtained and cultured. *β-catenin*-null ES cells were maintained on feeders in serum and LIF and exhibited a more flattened morphology than heterozygote controls (Fig5.5.1a). When cultured on gelatine they differentiated spontaneously and grew poorly (Fig5.5.1a). Addition of a MEK-inhibitor (PD03) enhanced self-renewal of heterozygous cells but appeared to promote differentiation of null cells into large, flattened cells that ceased proliferating (Fig5.5.1a). Viability was extremely poor when the cells were cultured in serum-free conditions with the cells adopting a dispersed morphology and increasing cell death (Fig5.5.1b). Cultures could not be maintained in any serum-free condition tested. The behaviour of these cells, in particular their response to MEK-inhibition, is inconsistent with the ES cell phenotype. The loss of β-catenin may have irreversibly changed the properties of these cells such that they no longer respond to signalling pathways in the same way as wild-type ES cells. Analysis of gene expression by real-time PCR confirms that *β-catenin*-null ES cells differ from wild-type ES cells (Fig5.5.1c). They express significantly lower levels of *Nanog* and elevated levels of *Fgf5* (Fig5.5.1c). *Oct4* levels were reduced approximately 2-fold. This may be consistent with an EpiSC phenotype (Brons et al., 2007; Tesar et al., 2007) or another differentiated derivative of ES cells. I conclude that the cells are not appropriate to test the requirements for β-catenin in 3i culture.

Conditional deletion of a floxed allele in *β-catenin* heterozygote cells (Fig5.5) provided an opportunity to test directly the effect of loss of β-catenin in the context of 3i. *β-catenin*$^{-/flox}$ ES cells were cultured in 3i and transfected with a plasmid encoding Cre. Transfected cells could be identified by expression of GFP transcribed downstream of Cre and translated from an IRES. 24 hours after transfection cells were sorted by flow cytometry for GFP expression and plated onto MEFs in serum and LIF or in 3i. The GFP- population efficiently formed colonies in 3i on MEFs as expected but the GFP+ population formed significantly fewer colonies (Fig5.5.1d). The colonies formed from the GFP+ population were morphologically distinct. They formed flat colonies rather than rounded up colonies and had poor cell-cell adhesion (Fig5.5.1e) perhaps as a result of the loss of adherens junctions (Bienz, 2005). These colonies grew poorly and could not be picked and expanded as clones. The reduction in colony forming ability and the apparent cessation in growth suggests that β-catenin is essential for growth in 3i. However, the cells also grew poorly in conditions intended to serve as a positive control, serum and LIF, making it difficult for us to interpret this experiment with confidence. It may be that it is not possible to generate *bona fide* ES cells lacking β-catenin. It would be informative to repeat this experiment and to include growth conditions that support the growth of EpiSCs to investigate the possibility that the loss of β-catenin promotes a conversion to this cell type.

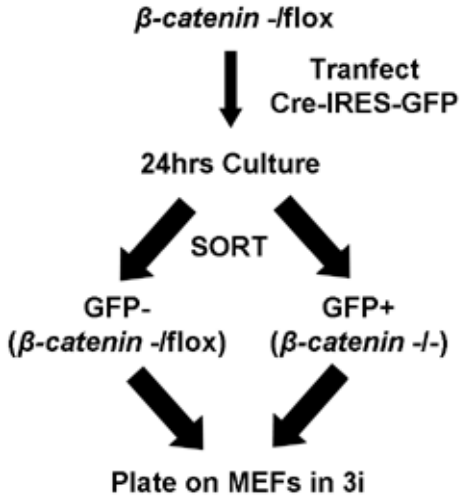


Fig5.5. Schematic showing strategy for isolation of *β-catenin*-null ES cells. ES cells carrying one null and one floxed *β-catenin* allele were transfected with a plasmid encoding Cre-recombinase and GFP, expressed from the same promoter. 24hrs later transfected cells were separated from non-transfected cells by flow cytometry for GFP expression. Transfected (GFP positive) cells are expected to have deleted the second *β-catenin* allele.GFP positive and negative populations were compared for their ability to grow on MEFs in 3i.

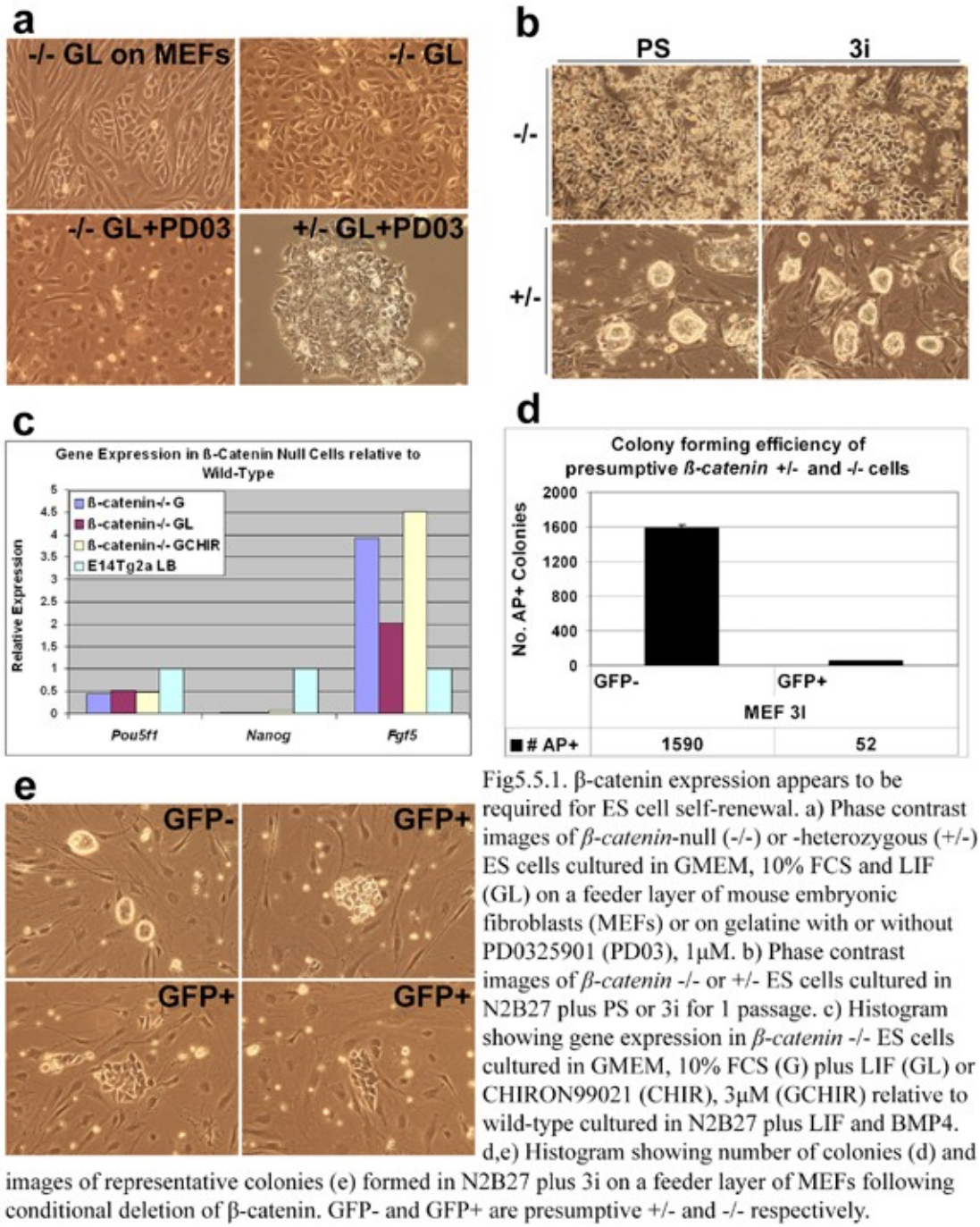


Fig5.5.1. β-catenin expression appears to be required for ES cell self-renewal. a) Phase contrast images of *β-catenin*-null (-/-) or -heterozygous (+/-) ES cells cultured in GMEM, 10% FCS and LIF (GL) on a feeder layer of mouse embryonic fibroblasts (MEFs) or on gelatine with or without PD0325901 (PD03), 1μM. b) Phase contrast images of *β-catenin* -/- or +/- ES cells cultured in N2B27 plus PS or 3i for 1 passage. c) Histogram showing gene expression in *β-catenin* -/- ES cells cultured in GMEM, 10% FCS (G) plus LIF (GL) or CHIRON99021 (CHIR), 3μM (GCHIR) relative to wild-type cultured in N2B27 plus LIF and BMP4. d,e) Histogram showing number of colonies (d) and images of representative colonies (e) formed in N2B27 plus 3i on a feeder layer of MEFs following conditional deletion of β-catenin. GFP- and GFP+ are presumptive +/- and -/- respectively.

5.2.3 Adherens Junctions

Adherens junctions are protein complexes occurring at cell-cell junctions that mediate cell-cell interactions (reviewed in (Bienz, 2005)). Cadherins are transmembrane proteins and essential components of adherens junctions. They dimerize with other cadherins on adjacent cells and are linked through catenins to the

actin cytoskeleton (reviewed in (Weis and Nelson, 2006)). E-cadherin (Ecad) is expressed in the oocyte and in all cells of the early embryo (Larue et al., 1994). It is required for compaction and for trophectoderm formation. Embryos lacking E-cadherin die around the time of implantation (Larue et al., 1994). *Ecad-/-* ES cells exhibit decreased cell-cell adhesion and fail to form aggregates in suspension culture but they retain expression of Oct4 and can form teratomas when injected into immuno-compromised mice (Larue et al., 1996). The teratomas display markers of differentiation but fail to form organized structures. β-catenin binds directly to cadherins and is thought to be essential for the interaction of adherens complexes with the actin cytoskeleton (reviewed in (Weis and Nelson, 2006)). The pool of β-catenin incorporated in these complexes is thought to be relatively stable and separate from the pool that interacts with the destruction complex and mediates canonical Wnt signalling (reviewed in (Bienz, 2005)). However, it remains a possibility that stabilisation of β-catenin through inhibition of GSK3 may influence adherens junctions. In fact, our observations that ES cells grown in the presence of CHIR form rounded up colonies, a phenotype indicative of increased cell-cell adhesion, suggests that this may be the case.

ES cells lacking E-cadherin were obtained (Larue et al., 1996). These cells adhere well to gelatine but assume a more dispersed morphology than wild-type controls (Fig5.6a). They are routinely cultured in serum plus LIF and upon transfer to serum-free conditions exhibit a degree of cell death. Seemingly, an initial adaptation period is required after which the cells grow robustly in 3i, 2i or LIF plus BMP4 (Fig5.6a,b). Failure to form the rounded up colonies observed in wild-type 3i cultures indicates that Ecad is required for this morphological change. To assess the effect of CHIR cells were transferred from 2i to PD03 or CHIR alone. They could be propagated in both conditions but grew poorly compared to 2i. In CHIR alone the cells assumed a more refractile morphology and tended to detach from the plate while in PD03 alone spontaneous differentiation was more evident (Fig5.6a). After 5 passages in 3i or in LIF plus BMP4 *Ecad*-/- cells remained positive for Oct4 and Nanog (Fig5.6b)**.** To assess the effect of the inhibitors quantitatively the cells were plated at clonal density in different culture conditions and the resulting colonies

stained for alkaline phosphatase after 6 days' growth. In standard growth conditions, serum plus LIF, colony forming efficiency was high (Fig5.6.1a) with most colonies having a core of AP-positive cells surrounded by a skirt of differentiation (Fig5.6.1b). In N2B27 alone very few colonies formed (Fig5.6.1a). Addition of either PD03 or CHIR enhanced the efficiency of colony formation and the 2i combination gave rise to undifferentiated colonies with the highest efficiency of all conditions tested (Fig5.6.1a). Morphologically, colonies formed in all serum-free conditions were smaller than in serum plus LIF and they tended to be dispersed although in LB or 2i colonies remained more compact (Fig5.6.1b).

In section 5.2.2 a positive effect of canonical Wnt signalling on colony formation in wild-type ES cells was observed. However, the downstream effectors remain unclear. Addition of Wnt3a to bulk cultures of *Ecad*-/- ES cells had little discernible effect (Fig.5.6.1c). Colony forming efficiency was not enhanced by the addition of Wnt3a in the presence or absence of PD03 (Fig5.6.1a) raising the possibility that the effect of Wnt3a on wild-type cells is mediated through an increase in adherens junction-mediated cell-cell adhesion. No consistent morphological difference was apparent between colonies grown with or without Wnt3a (Fig5.6.1c). Batch-to-batch variation in Wnt3a activity is possible. To test that the Wnt3a used in this experiment was active, wild-type ES cells were cultured side-by-side short term in N2B27 alone or plus Wnt3a. While in N2B27 alone most cells died and only a few colonies persisted, in the presence of Wnt3a cell survival was enhanced and a morphologically heterogeneous culture developed (Fig5.6.1d). The difference clearly demonstrated that the Wnt3a was active. To eliminate the possibility that prior adaptation to Wnt3a culture conditions was required for an effect to be observed on colony formation *Ecad*-/- cells were cultured for two passages in PD03 plus Wnt3a prior to plating at clonal density. Furthermore, media was changed after three days to control for a loss of Wnt3a activity over the course of the 6 day experiment. Cells were plated in the presence of PD03 in the presence or absence of CHIR or Wnt3a. While the addition of CHIR to cultures enhanced the number of colonies formed and the proportion that were totally undifferentiated, Wnt3a had no significant effect (Fig5.6.1e). I conclude from these findings that increased cell-cell adhesion is not critical to ES cell

maintenance in 3i but that it may have a role in the promotion of self-renewal by Wnt3a. To be confident of this it would be necessary to rescue the response to Wnt3a by re-expressing *Ecad*. Similarly, reduced Ecad function in wild-type cells may ablate the response to Wnt3a (Fig5.4c). These data also raise the question of whether expression of stabilized β-catenin would have any effect on ES cells lacking Ecad.

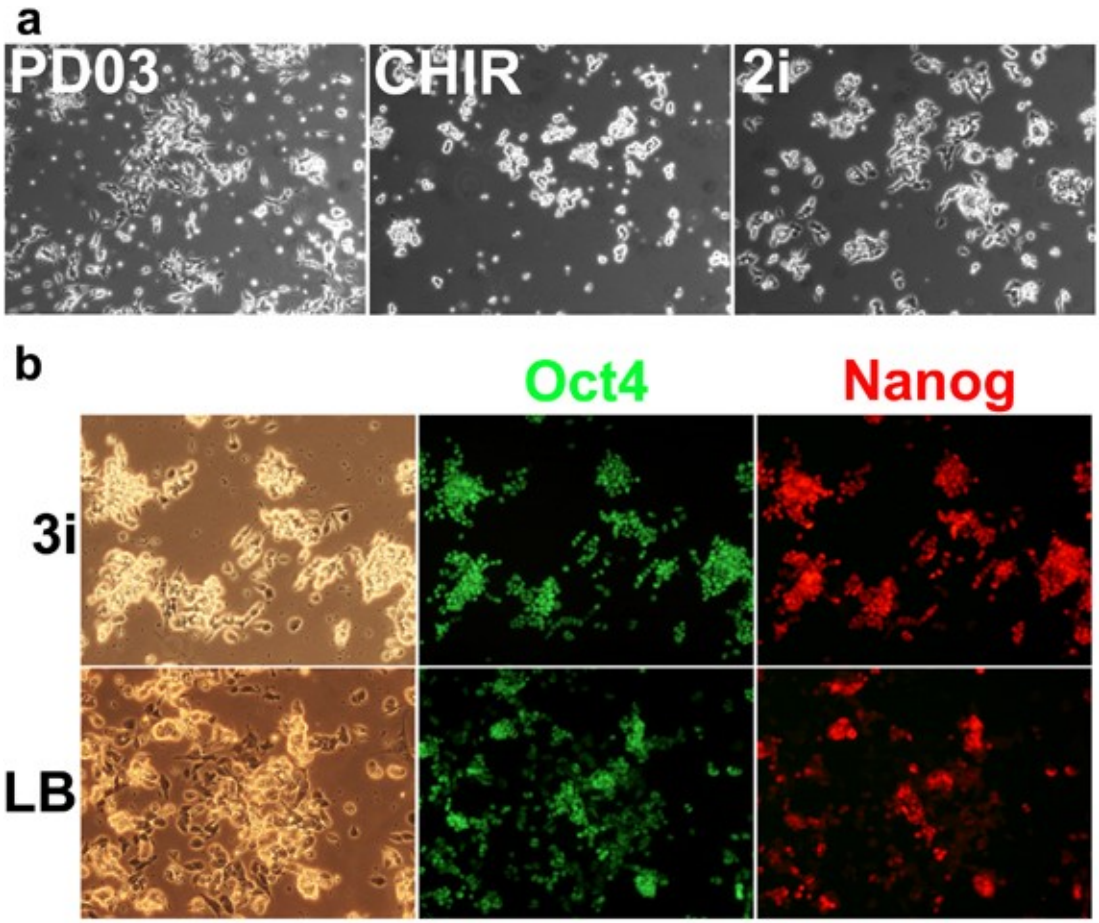


Fig5.6. ES cell culture in 3i/2i does not require adherens junctions. a) Phase contrast images of *E-cadherin*-null (*Ecad*-/-) ES cells cultured in N2B27 plus PD0325901 (PD03), 1μM; CHIRON99021 (CHIR), 3μM or 2i for 3 (PD03, CHIR) or 7 (2i) passages. b) *Ecad*-/- ES cells were cultured in N2B27 plus 3i or LIF and BMP4 (LB) for 5 passages, fixed and immunostained for Oct4 and Nanog.

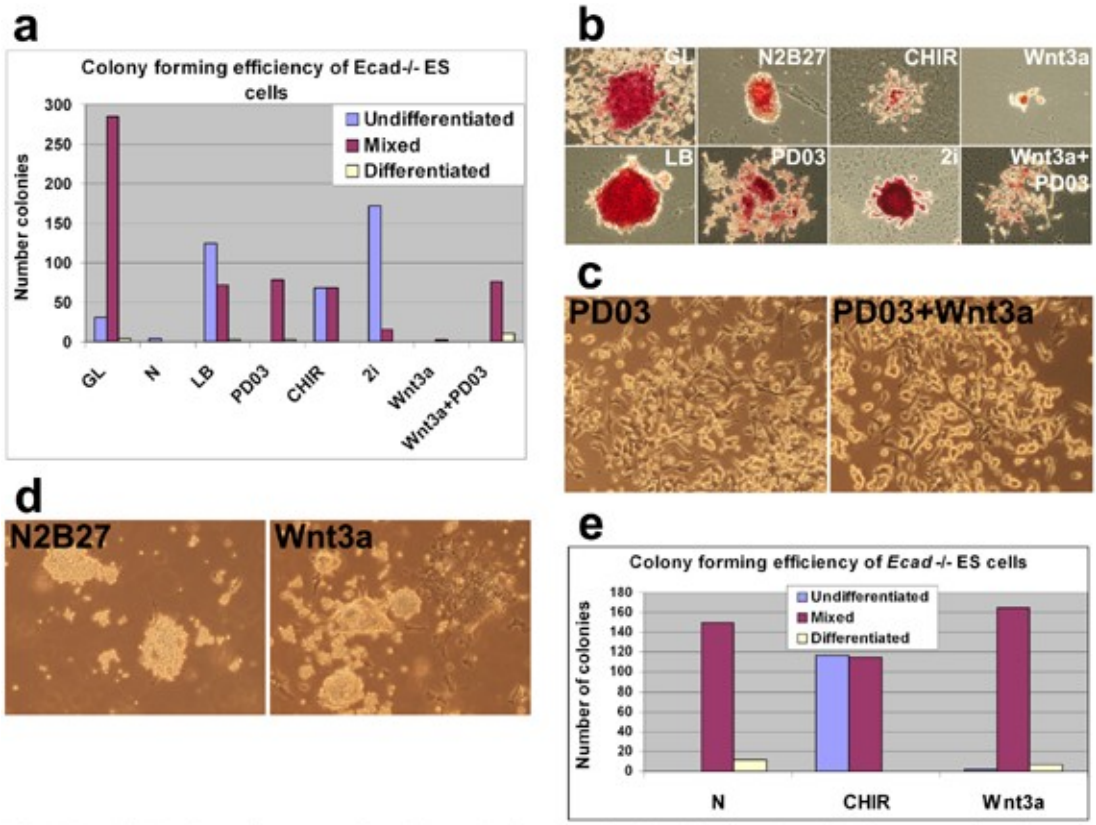


Fig5.6.1. 3i/2i culture is not mediated through increased cell-cell adhesion. a) Histogram showing colony forming efficiency of *E-cadherin*-null (*Ecad*-/-) ES cells plated at clonal density in GMEM, 10% FCS plus LIF; N2B27 alone (N) or plus LIF and BMP4 (LB); PD0325901 (PD03), 1μM; CHIRON99021 (CHIR), 3μM; 2i; Wnt3a, 100ng/ml; or Wnt3a, 100ng/ml plus PD03, 1μM. Colonies were scored as alkaline phosphatase-positive (undifferentiated), -negative (differentiated) or mixed. b) Images of colonies from each condition in (a). c) Phase contrast images of *Ecad*-/- ES cells after 2 passages in N2B27 plus PD03, 1μM or Wnt3a, 100ng/ml. d) Phase contrast images of E14Tg2a ES cells cultured in N2B27 alone or plus Wnt3a, 100ng/ml. e) Histogram showing colony forming efficiency of *Ecad*-/- ES cells plated at clonal density in N2B27 plus PD03, 1μM alone (N) or plus CHIR, 3μM or Wnt3a, 100ng/ml. Cells were cultured for 2 passages in Wnt3a plus PD03 prior to plating.

5.2.4 Candidate Identification and Analysis of the role of CHIRON99021

The data presented in chapter 4 show that ES cells cultured in PS or PD03 are partially prevented from differentiating but that efficient propagation and clonal self-renewal only occurs if either LIF or CHIR are included in the culture media. CHIR eliminates residual neural differentiation but also has a clear effect on the survival and proliferation of ES cells. This is perhaps most clearly seen in the enhancement of colony formation of Nanog-overexpressing cells (Fig5.8a). I reasoned that known

targets of GSK3 and genes identified in the literature that enhance self-renewal were candidate downstream targets of CHIR.

5.2.4.1 cMyc

Myelocytomatosis oncogene (Myc or cMyc) is a basic helix-loop-helix leucine zipper (bHLH-LZ) TF that controls the expression of target genes through its interaction with co-factors (reviewed in (Dang et al., 2006)). cMyc interacts with Max to activate transcription but the Myc:Max heterodimers can also inhibit transcription through interaction with Miz1. Mad can also bind Max to inhibit transcription and the relative levels of cMyc and Mad influence the activity of the ubiquitously expressed Max. Global analyses of the influence of cMyc on gene expression suggest it may regulate up to 15% of the genes in the genome. cMyc target genes can be grouped according to function and include regulators of metabolism, protein biosynthesis, the cell cycle, cell adhesion and the cytoskeleton (Dang et al., 2006).

Several properties of cMyc and its regulation made it an interesting candidate for study in the context of 3i and as a possible downstream target of GSK3 inhibition. As a known promoter of cell growth and proliferation (reviewed in (Sears, 2004)) elevated cMyc expression might promote the growth of ES cells, particularly in the context of PS where proliferation was observed to be compromised (see chapter 4). cMyc has also been implicated in the regulation of stem cell self-renewal and differentiation (Murphy et al., 2005). Of particular relevance for our studies is the apparent demonstration that cMyc is a transcriptional target of STAT3 and that forced expression of cMyc can relieve ES cells from their dependence on LIF-STAT3 signalling (Cartwright et al., 2005a). The authors of this work also suggested that cMyc represents a common target of canonical Wnt signalling and LIF-STAT3 and might be a point of convergence in their ability to promote self-renewal. It should be noted, however, that *cMyc*-null mice do not die until after the formation of the pluripotent epiblast (Trumpp et al., 2001) and that *cMyc*-null ES cells can be derived and maintained, exhibiting only a slight decrease in growth rate (Baudino et

al., 2002). However, it is likely that cMyc exhibits redundancy with the related N-Myc. Testing the Myc-dependence of ES cells would require the derivation of double knockout lines (Murphy et al., 2005).

cMyc is regulated at the level of transcription and also at the level of protein stability (Fig5.7). cMyc transcription has been shown to increase in response to mitogenic stimuli through activation of the ERK signalling cascade. It is also a target of β-catenin (He et al., 1998), placing it downstream of Wnt or of GSK3 inhibition. Given that in 3i ERK activation is blocked while GSK3 is inhibited it is conceivable that their contrasting effects on *cMyc* transcription might act to balance one another and maintain a critical level of *cMyc*. cMyc is also highly regulated at the level of protein stability (Sears, 2004; Dai et al., 2006), with significant changes in proteins levels sometimes occurring in the absence of any change in mRNA levels. Phosphorylation of cMyc at two sites in its N-terminus, Thr58 and Ser62 (Lutterbach and Hann, 1994; Pulverer et al., 1994) regulates its stability. Ser62 phosphorylation stabilizes the protein while Thr58 phosphorylation promotes its ubiquitin-dependent proteolysis. Thr58 phosphorylation is dependent on prior phosphorylation at Ser62 and ubiquitylation requires removal of the Ser62 mark by phosphatases (reviewed in (Sears, 2004)). Ser62 is phosphorylated by ERK in response to activation of the Ras-Raf-MEK-ERK cascade while Thr58 is phosphorylated by GSK3 (Gregory et al., 2003). Serum stimulation of quiescent cells leads to rapid accumulation of cMyc. It is thought this occurs through activation of ERK and inhibition of GSK3 through PI3K-PKB-mediated phosphorylation, leading to the accumulation of cMyc stabilized by phosphorylation on Ser62 but not Thr58 (Sears, 2004). The inhibitors in 3i are predicted to influence cMyc protein stability both positively (CHIR) and negatively (PS) (Fig5.7).

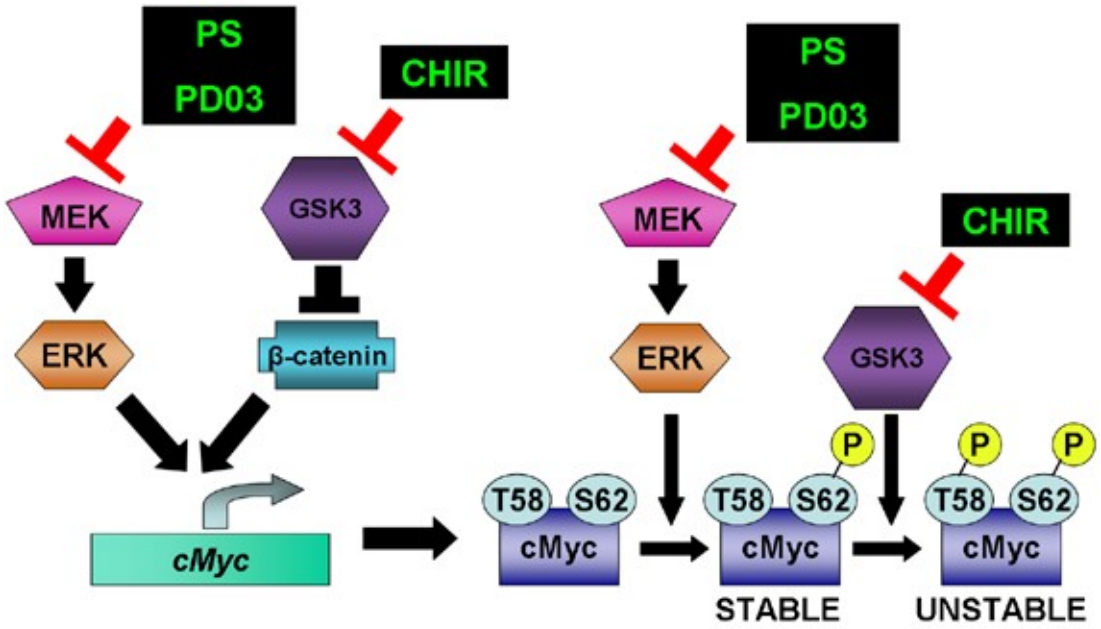


Fig5.7. Predicted opposing effects of signalling downstream of ERK and GSK3 on *cMyc* transcription and protein stability. *cMyc* transcription is positively regulated by ERK signalling and by β-catenin. Transcription is therefore predicted to be negatively regulated by PS or PD0325901 (PD03) and positively regulated by CHIRON99021 (CHIR). Phosphorylation of cMyc on Ser62 (S62) by ERK increases protein stability while subsequent phosphorylation by GSK3 on Thr58 (T58) decreases stability. Protein stability is therefore predicted to be negatively regulated by PS or PD03 and positively regulated by CHIR.

The level of cMyc expression was examined at both the transcript and protein level in 3i as compared to other growth conditions and in different combinations of the three inhibitors. In steady state conditions the *cMyc* transcript was decreased in both 3i and PS (Fig5.7.1a) as compared to cells grown in serum and LIF, LIF and BMP4, or CHIR alone. These results indicate that transcript levels are not significantly affected by the absence of serum ((Fig5.7.1a) compare GL with LB) or by the presence of LIF ((Fig5.7.1a) compare N with LB). However, in PS or 3i, the conditions where ERK activity is significantly down-regulated, *cMyc* transcript levels were significantly reduced. The presence of CHIR (either in 3i or alone) did not elevate transcript levels, as would be expected if *cMyc* was responsive to canonical Wnt signalling in ES cells (He et al., 1998), and if anything lowered the level of *cMyc* transcript (Fig5.7.1a). The changes in *cMyc* occurred independently of changes in markers of pluripotency, *Oct4* and *Nanog*, indicating that differing *cMyc* levels did not reflect differentiation. To ascertain if *cMyc* responds directly to PS acute response assays were performed. ES cells were starved of cytokines and then

treated with inhibitors or cytokines for one hour. *Egr1* served as a control for activation (LIF) or inhibition (PS and 3i) of ERK signalling. Similar levels of *Oct4* and *Nanog* expression in all conditions indicate that there was little variation in the proportion of ES cells in the population. There was no significant change in *cMyc* in response to LIF, CHIR or Wnt3a but levels were significantly down-regulated in response to PS or 3i (Fig5.7.1b) showing that *cMyc* transcription responds rapidly to inhibition of the FGF-MEK-ERK signal and is likely to be a direct target of this signal. Note that activation of known β-catenin target genes, *Axin2* and *T*, was not observed in this time-frame (Fig5.7.1c) possibly because of the time needed for β-catenin to accumulate in the nucleus. I conclude from these results that levels of *cMyc* transcript decrease in response to PS and that this is not affected by the presence of CHIR. Furthermore, there is no evidence of a response to LIF as has previously been reported. However, it was not examined in the context of short-term stimulation experiments (Cartwright et al., 2005a). Interestingly, Wnt3a activated transcription of the immediate early genes (IEGs), *Egr1*, *c-Fos* and *JunB*, indicating that it may have effects independent of the canonical Wnt pathway (see discussion).

Mice lacking N-Myc die around E11.5 (Charron et al., 1992; Stanton et al., 1992), beyond the point when the pluripotent epiblast has been established. Furthermore, *N-Myc*-null ES cells have been reported (Sawai et al., 1991; Malynn et al., 1995) indicating that N-Myc is not essential for pluripotency. However, N-Myc may well exhibit functional redundancy with cMyc and it is expressed in ES cells (Murphy et al., 2005). The response of *N-Myc* in the same experiments designed to assess the response of *cMyc* was examined. The *N-Myc* transcript responded similarly to *cMyc*, although the differences in expression tended to be smaller (Fig5.7.1a,b), suggesting that it is subject to the same regulatory pathways in ES cells.

The level of post-translational control exerted on cMyc means that it cannot be assumed that protein levels reflect the transcript levels faithfully. The effect of GSK3 inhibition on cMyc protein levels was of particular interest given that GSK3 phosphorylates Thr58 of cMyc and targets it for proteolysis (Gregory et al., 2003). Inhibiting GSK3 would be expected to lead to an increase in protein levels from an

equivalent amount of transcript. Attempts to assess the level of cMyc protein by western blotting were unconvincing because of the poor specificity of several antibodies tested. Therefore, immunoprecipitations (IPs) of cMyc were performed on lysates from ES cells cultured under different growth conditions. Subsequent immunoblotting with the same cMyc antibody as was used for IP revealed a clean band migrating in the correct size range for cMyc. The absence of a corresponding band in isotype controls (Fig5.7.1d,e, MOCK) shows that the band detected is specific for cMyc. cMyc protein levels appeared to be decreased in serum-free conditions (Fig5.7.1d, compare GL to N2B27) but notably they were further decreased in PS (Fig5.7.1d), in agreement with the transcript data (Fig5.7.1a). Addition of LIF or CHIR to PS failed to increase levels of cMyc (Fig5.7.1d,e) while CHIR alone had little or no effect (Fig5.7.1d, compare CHIR to N2B27). Note, however, that levels are higher in CHIR alone than in PS or 3i suggesting that lower levels of transcription resulting from ERK inhibition and/or decreased protein stability upon loss of Ser62 phosphorylation are the dominant effects on cMyc protein levels in ES cells. I conclude that cMyc protein levels are decreased in response to PS and do not respond in this context to either LIF or GSK3 inhibition. As there is a clear phenotypic response, the efficient self-renewal of ES cells in either PS+LIF or 3i, elevated levels of cMyc are not critical for self-renewal in these culture conditions and ES cells respond to LIF and to GSK3 inhibition through pathways that do not affect cMyc expression.

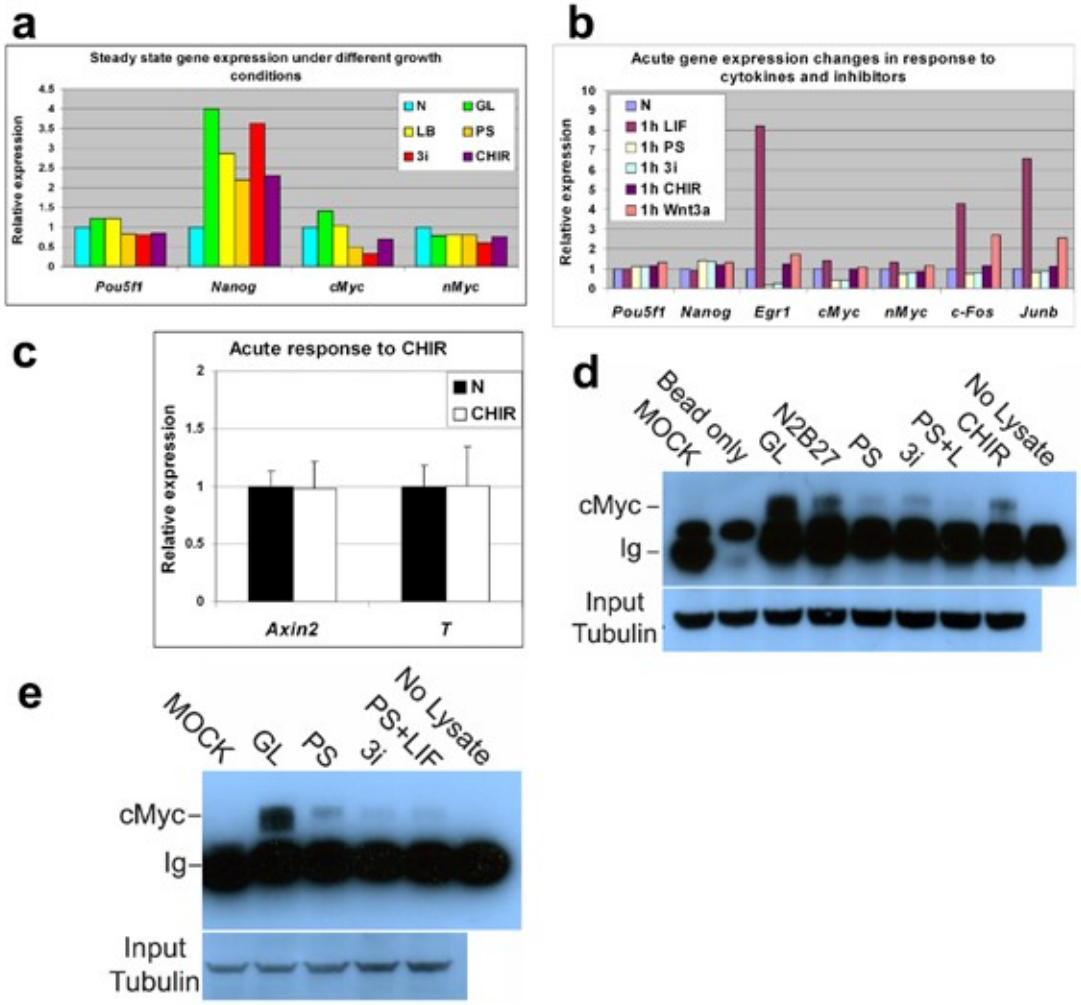


Fig5.7.1. cMyc is downregulated by MEK inhibition and does not respond to LIF or CHIR. a) Histogram showing relative gene expression in E14Tg2a ES cells cultured for 24 hours in N2B27 alone or plus LIF and BMP4 (LB); PS; 3i; or CHIRON99021 (CHIR), 3μM; or in GMEM, 10% FCS plus LIF (GL). Gene expression was normalised to β-actin for each sample. b) Histogram showing relative gene expression in E14Tg2a ES cells cultured for 6 hours in N2B27 alone followed by 1 hour in N2B27 alone (N) or plus LIF; PS; 3i; CHIR, 3μM; or Wnt3a, 100ng/ml. Gene expression was normalised to β-actin for each sample. c) Histogram showing relative gene expression in E14Tg2a ES cells cultured for 6 hours in N2B27 alone followed by 1 hour in N2B27 alone (N) or plus CHIR, 3μM. d,e) E14Tg2a ES cells were cultured for 24 hours in GL or N2B27 alone or plus PS; 3i; PS+LIF (PS+L); or CHIR, 3μM, lysed and immunoprecipitations performed for cMyc. Lysates were subjected to SDS-PAGE and western blots interrogated with cMyc antibody. MOCK samples were treated identically to GL but IPs were performed with Tubulin antibody to serve as an isotype control. 'Bead only' controls were as GL but no antibody was added. No Lysate controls were as GL but lysis buffer was added instead of lysate.

5.2.4.2 Nanog

Unlike regulation of MEK-ERK signalling there is little evidence that GSK3 inhibition should affect Nanog expression. It has been suggested that β-catenin can

promote *Nanog* expression through interaction with Oct4 (Takao et al., 2007) but the experiments in that paper fail to address the direct regulation of the *Nanog* promoter. However, the proposed role of canonical Wnt signalling in self-renewal (Sato et al., 2004; Liu et al., 2006; Ogawa et al., 2006) and the many possible downstream effects of GSK3 (reviewed in (Doble and Woodgett, 2003)) inhibition make Nanog worth testing as a candidate target of CHIR.

In chapter 4 I showed that Nanog levels were elevated in 3i but that Nanog did not appear to respond directly to CHIR. It seems, therefore, that the short-term maintenance of Nanog expression as seen by immunoblotting (see chapter 4) and from the propagation of ES cells in CHIR alone (Fig5.1a) that GSK3 inhibition maintains Nanog expression indirectly, presumably by inhibiting differentiation. I reasoned that if the effect of CHIR is independent of the direct regulation of Nanog that the effect would be evident in ES cells overexpressing Nanog or lacking Nanog altogether.

When Nanog overexpression was originally observed to support LIF-independent self-renewal it was clearly demonstrated that the addition of LIF further enhanced self-renewal (Chambers et al., 2003). In a similar test of the function of the inhibitors clonal assays were performed with Nanog-overexpressing cells. The efficiency of self-renewal was measured as the number of colonies formed and the proportion staining positive for the pluripotency marker, alkaline phosphatase (AP), when the cells were plated at low density (60cells/cm^2) and allowed to form colonies for 6-7 days. Formation of undifferentiated and mixed colonies in N2B27 alone is characteristic of ES cells overexpressing Nanog (Fig5.8a). In this assay the addition of CHIR, alone or in the context of 3i, significantly enhanced the number of colonies formed by Nanog overexpressing cells (Fig5.8a), indicating that CHIR has potent effects independent of Nanog regulation. The colonies formed in the presence of CHIR also tended to be larger and to have a more regular, rounded up morphology, staining intensely for AP (Fig5.8b). Interestingly, the presence of PS had little effect on Nanog overexpressing cells either alone or in the context of 3i (Fig5.8a). As our working hypothesis is that PS functions primarily to prevent differentiation it is

likely that in the context of Nanog overexpression, where differentiation is already effectively blocked, PS no longer has a positive effect on self-renewal.

ES cells lacking Nanog also retain their response to CHIR. *Nanog*-null (44Cre6) ES cells retained *Nanog*-GFP expression in the presence of CHIR while it was lost in cultures in N2B27 alone (Fig5.8c). Colonies formed in CHIR alone tended to round up and detach from the plate although they retained expression of the Nanog reporter (Fig5.8c). Growth rate was significantly enhanced in 2i as compared to PD03 alone indicating that *Nanog*-null ES cells respond to CHIR similarly to wild-type ES cells. Notably, spontaneous differentiation remained high in all combinations of inhibitors tested (as observed in chapter 4). These results, together with those presented in chapter 4, indicate that Nanog-null ES cells respond to inhibition of GSK3 and the ERK pathway by increasing self-renewal but that in the absence of Nanog spontaneous differentiation remains high.

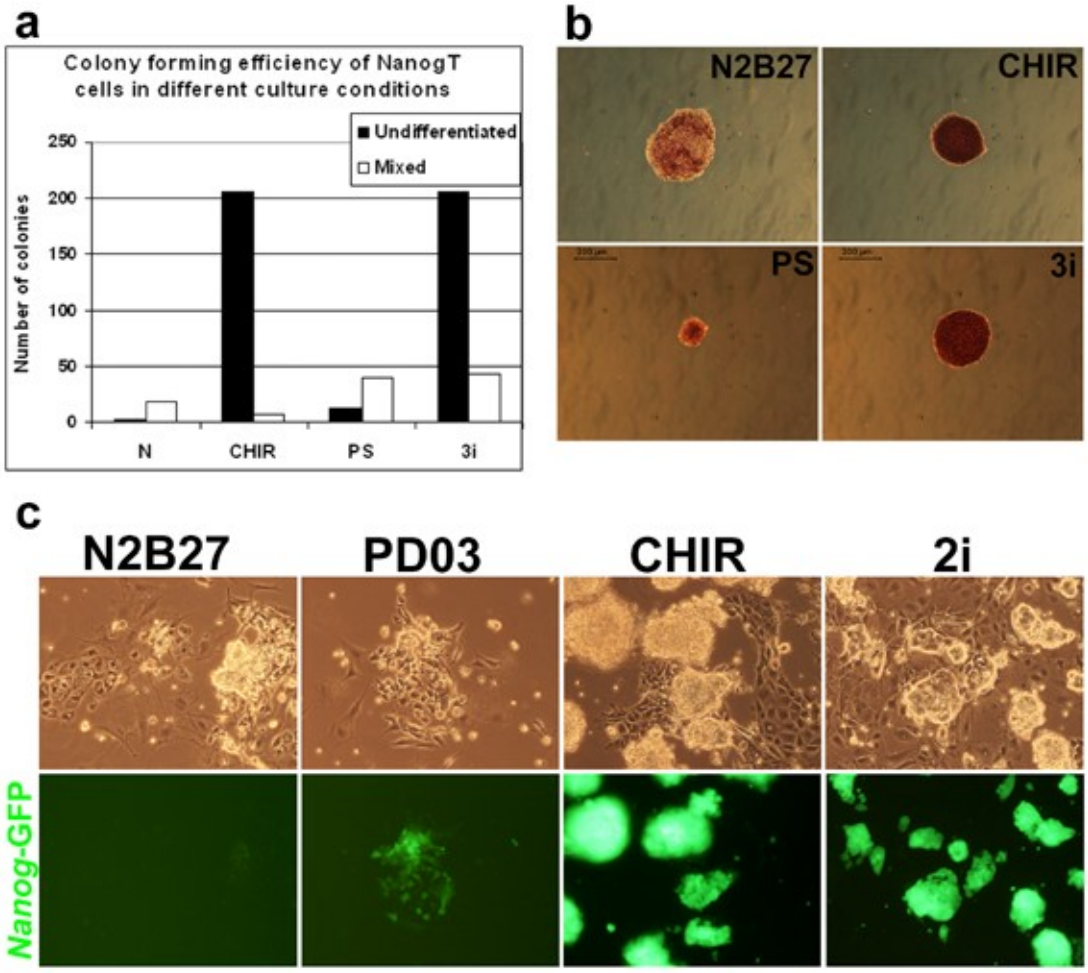


Fig5.8. The effect of CHIR is Nanog-independent. a) Histogram showing number of alkaline-phosphatase positive (undifferentiated) and mixed colonies formed by NanogT (Nanog-overexpressing) ES cells plated at clonal density in N2B27 alone (N) or plus CHIRON99021 (CHIR), 3μM; PS; or 3i. b) Representative images of alkaline phosphatase-stained colonies from each condition in (a). c) Phase contrast and *Nanog*-GFP images of 44cre6 (*Nanog*-null) ES cells cultured in N2B27 alone or plus PD0325901 (PD03), 1μM; CHIR, 3μM; or 2i for 2 passages.

5.2.4.3 Klf4

Kruppel-like factor 4 (Klf4) is a zinc finger TF. It is expressed in ES cells and was identified as a putative target gene of STAT3 signalling (Li et al., 2005). A microarray expression analysis based on the hyper LIF-sensitive $Shp2^{\Delta 46\text{-}110}$ ES cells identified *Socs3* and *Klf4* as targets. SOCS3 overexpression induced differentiation as expected for an inhibitor of STAT3 activation but Klf4 overexpression appeared to inhibit differentiation. Forced *Klf4* expression enhanced the formation of secondary EBs, implying the persistence of stem cells, which was supported by the

finding that Oct4 expression was much higher in day 6 EBs from Klf4-expressing ES cells than controls. It has since been shown that Klf4 cooperates with Oct4 and Sox2 to activate the target gene *Lefty1* (Nakatake et al., 2006). A *Lefty1* luciferase reporter is not activated in a heterologous cell type when cotransfected with Oct4 and Sox2 in contrast to other well defined Oct4 targets. However, the analysis of candidate genes showed that Klf4 was capable of activating the *Lefty1* reporter. Activation was enhanced in the presence of Oct4 and Sox2 demonstrating cooperative activation of the *Lefty1* promoter. Microarray analysis of putative Klf4 targets, revealed by *Klf4* shRNA, and comparison to target genes positively regulated by Oct4 revealed overlap between targets. This raises the possibility that there is a set of genes co-regulated by Klf4 and Oct4 (Nakatake et al., 2006). Finally, in the light of the recent demonstration that somatic cells can be reprogrammed to pluripotency by transfection with 4 TFs (Takahashi and Yamanaka, 2006), one of which is Klf4, it seems that Klf4 has a significant role in the regulation of pluripotency. While the precise roles of Klf4 in ES cell self-renewal or in reprogramming await further study there is enough evidence to make it an interesting candidate for this thesis.

Klf4 expression levels were examined in ES cells cultured in different conditions together with the related Klf family member, *Klf2*, which demonstrates similar activation of the *Lefty1* reporter to Klf4 (Nakatake et al., 2006). *Klf2* expression was slightly higher in steady state 3i culture than in LB while *Klf4* appeared to be expressed at slightly higher levels in LB (Fig5.9a). Both were down-regulated in PS which may reflect differentiation as suggested by the lower expression levels of pluripotency-associated genes (Fig5.9a). These small changes in transcript level tell us very little about the regulation of Klfs so their response to short-term exposure to the inhibitors or to LIF was tested (Fig5.9b). Activation of *Socs3* and *Egr1* serve as positive controls for activation of STAT3 and ERK respectively. Down-regulation of *Egr1* in response to PS confirms that ERK activity was reduced under this condition. Targets of canonical Wnt signalling, *Axin2*, *Cdx1* and *T*, were upregulated in response to 8 hours exposure to CHIR confirming that this pathway was activated (Fig5.9c). *Klf2* showed a small but reproducible upregulation in response to CHIR (8 hours) (Fig5.9c) while *Klf4* levels increased in response to LIF (1 hour) (Fig5.9b) in

agreement with a previous study (Li et al., 2005). Neither gene responded to PS (Fig5.9b).

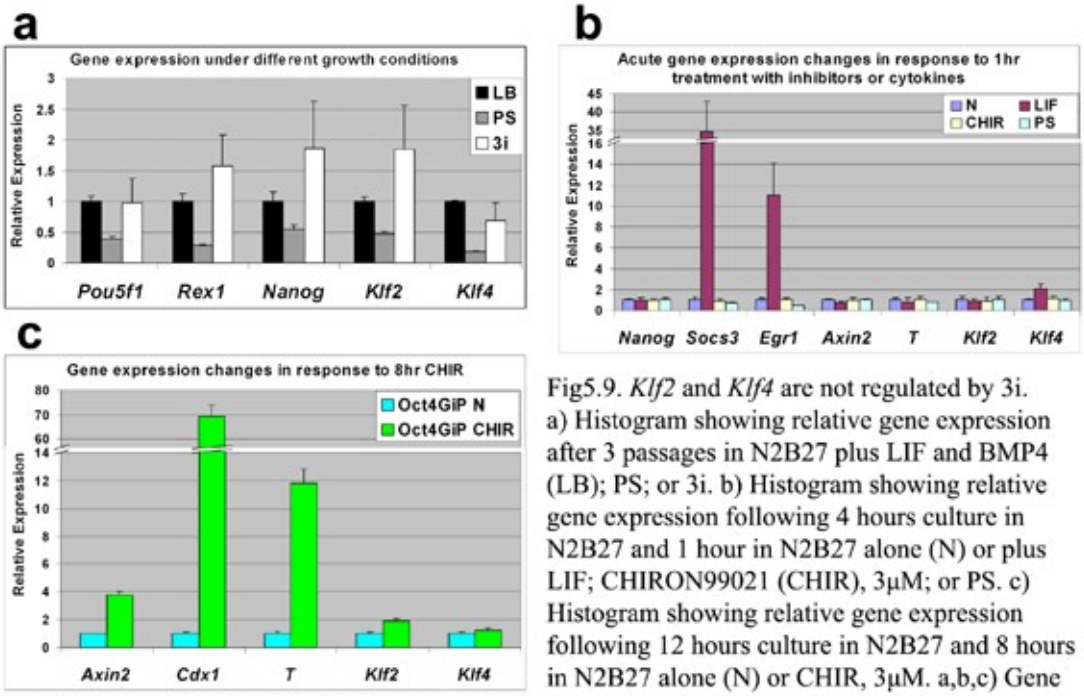


Fig5.9. *Klf2* and *Klf4* are not regulated by 3i. a) Histogram showing relative gene expression after 3 passages in N2B27 plus LIF and BMP4 (LB); PS; or 3i. b) Histogram showing relative gene expression following 4 hours culture in N2B27 and 1 hour in N2B27 alone (N) or plus LIF; CHIRON99021 (CHIR), 3μM; or PS. c) Histogram showing relative gene expression following 12 hours culture in N2B27 and 8 hours in N2B27 alone (N) or CHIR, 3μM. a,b,c) Gene expression was normalised to β-actin for each sample. Error bars represent standard deviation of the mean for 3 biological replicates.

To test a possible role for Klfs Klf4 was overexpressed in ES cells. Linearised plasmind DNA encoding *Klf4* under the control of a CAG promoter which also drives expression of a hygromycin resistance cassette was transfected into E14Tg2a ES cells. Hygromycin-resistant clones were selected for in N2B27 alone, PS or 3i. Resistant, alkaline phosphatase-positive colonies emerged with high efficiency in 3i and with lower efficiency in N2B27 and PS (Fig5.10a). This contrasts with empty vector transfections where no colonies can be derived in N2B27 alone (Fig5.10a). Colonies were picked for expansion in all conditions and the remainder of the 3i plate 'pooled'. Colonies in N2B27 or PS were capable of limited expansion suggesting that Klf4 was capable of supporting cytokine-independent self-renewal to a degree. Pooled Klf4-expressing cells were cultured in N2B27 alone, PS or 3i. Self-renewal in 3i was indistinguishable from wild-type cultures (Fig5.10b). In N2B27 alone the cultures expanded more than wild-type controls but accumulated differentiated cells (Fig5.10b). In PS spontaneous differentiation proceeded as

observed for wild-type ES cells with both neural (Fig5.10b, lower left) and non-neural (Fig5.10b, lower right) differentiation evident. I conclude that forced expression of Klf4 has a partial self-renewal phenotype but does not wholly reproduce the effects of either LIF or CHIR. These results are supported by the efficiency of colony formation at clonal density (Fig5.10c). In N2B27 alone very few colonies form while in PS a slightly higher number of colonies with a range of morphologies were observed (Fig5.10c). In LIF and BMP4 or in 3i large, undifferentiated colonies formed efficiently (Fig5.10c,d) whereas in CHIR alone fewer colonies formed (Fig5.10c) and had a tendency to exhibit more differentiation (Fig5.10d). Klf2 has been stably expressed by other members of the lab and found to have a similar phenotype to Klf4 (J. Hall, unpublished). The results described here suggest that stable expression of Klf4 can prolong the persistence of ES cells in the absence of cytokines but for efficient self renewal optimal growth conditions (LIF and BMP4 or 3i) are still required. While the possibility remains that the Klfs in part mediate the effect of LIF or CHIR they are clearly not the only targets promoting self-renewal. To test their requirement for 3i culture would require their genetic deletion or knockdown. These are experiments we plan to pursue.

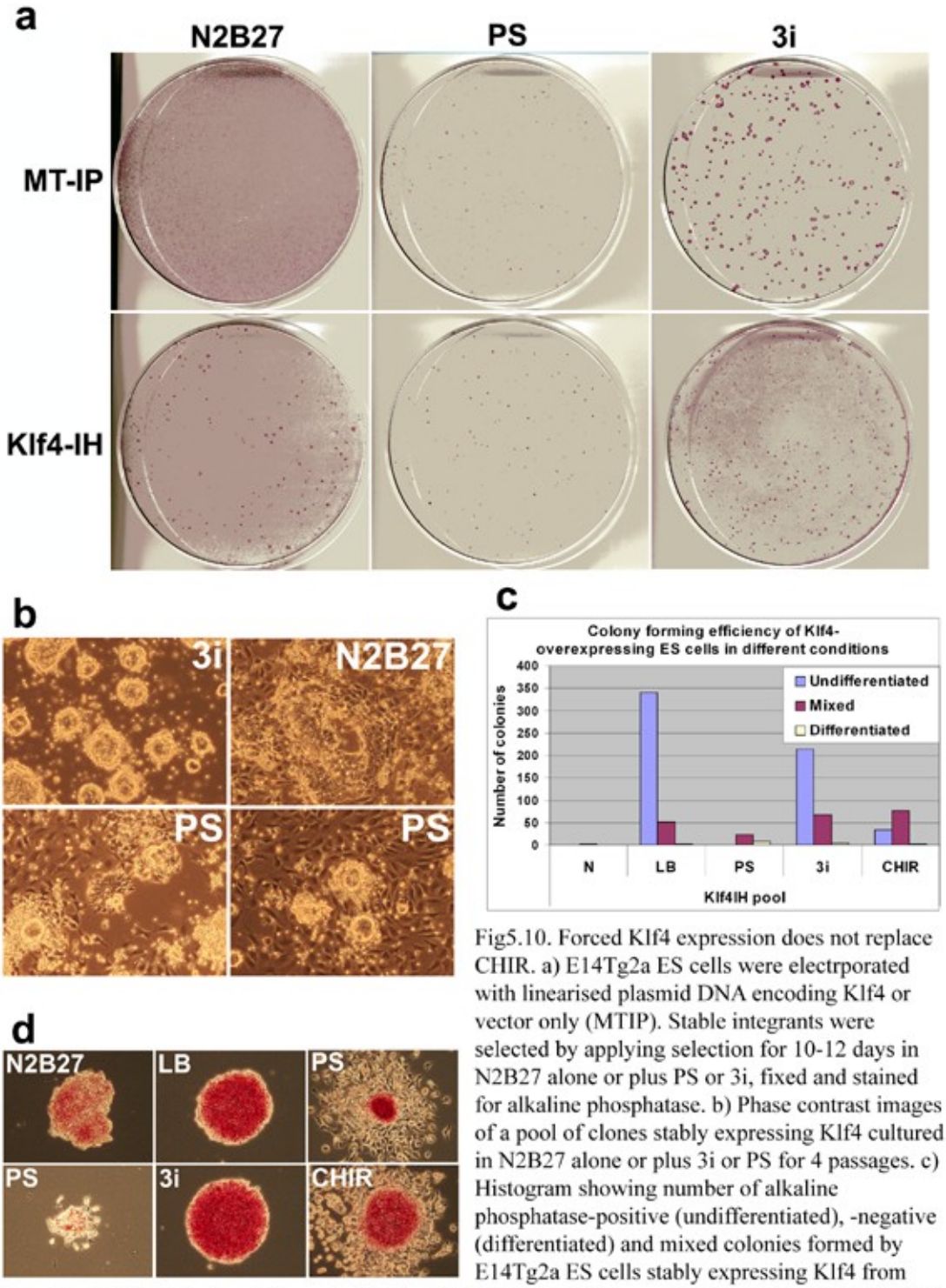


Fig5.10. Forced Klf4 expression does not replace CHIR. a) E14Tg2a ES cells were electrporated with linearised plasmid DNA encoding Klf4 or vector only (MTIP). Stable integrants were selected by applying selection for 10-12 days in N2B27 alone or plus PS or 3i, fixed and stained for alkaline phosphatase. b) Phase contrast images of a pool of clones stably expressing Klf4 cultured in N2B27 alone or plus 3i or PS for 4 passages. c) Histogram showing number of alkaline phosphatase-positive (undifferentiated), -negative (differentiated) and mixed colonies formed by E14Tg2a ES cells stably expressing Klf4 from 600 plated in N2B27 alone (N) or plus LIF and BMP4 (LB); PS; 3i; or CHIRON99021 (CHIR), 3μM. d) Images of colonies from (c).

5.2.4.4 Other pluripotency-associated genes

A trawl of the literature quickly unearths a large number of genes that have been implicated in ES cell self-renewal. They are usually identified on the basis of expression profile, microarray data, functional screening or some combination of the above. I have identified a few genes whose behaviour, as described in the literature, suggests that they might fulfil the role played by CHIR in 3i.

In 2006 a paper was published describing a screen undertaken to identify genes implicated in self-renewal (Ivanova et al., 2006). Genes rapidly down-regulated during retinoic acid-induced differentiation were identified by microarray analysis. From these, 65 genes were selected, consisting primarily of TFs and DNA-binding proteins and a further 5 genes were included from a previous study. shRNA knockdown vectors were designed for all 70 genes. Lentiviral vectors were used that expressed the shRNA from one promoter and GFP from another allowing transfected cells to be identified. Transfected (GFP+) cells were mixed at a ratio of 4:1 with non-transfected (GFP-) cells. The mixture was cultured in self-renewing conditions and the proportion of GFP+ cells identified at each passage. Cultures with a decreasing proportion of GFP+ cells represented shRNA vectors that impaired self-renewal. 10 of the 70 shRNAs tested had this effect and were selected for further analysis. Ultimately 7 genes were identified whose down-regulation caused morphological differentiation and the loss of alkaline phosphatase. Among them were *Oct4*, *Sox2* and *Nanog*, as would be expected. The others were the TFs *Tbx3* and *Esrrb*, the Akt1 co-factor *Tcl1* and the uncharacterized ES-specific gene *Dppa4*. Differentiation was only shown to occur in the context of LIF withdrawal in serum-containing conditions so it remains a possibility that the differentiation observed is context-specific. Constitutive expression of any of the 7 genes failed to prevent differentiation in EBs although some appeared to bias contribution to certain lineages. Interestingly, Nanog overexpression could rescue the differentiation observed upon knock-down of *Esrrb*, *Tbx3*, *Tcl1* or *Dppa4*. This indicates that these genes are not functioning down-stream of Nanog to prevent differentiation but also implies that their requirement for self-renewal is not absolute. Another interesting phenotype was observed upon deletion of the TF *Zfx* from ES cells (Galan-Caridad et al., 2007). ES cells lacking *Zfx* could be maintained and continued to express Oct4, Nanog and alkaline

phosphatase but exhibited reduced clonogenicity, slower growth and increased apoptosis. Furthermore, Zfx was proposed to regulate positively the expression of *Tbx3* and *Tcl1*. The expression levels of *Zfx*, *Tbx3* and *Tcl1* were examined in the context of 3i and response to the inhibitors. The antiapoptotic gene *Bcl2* and the related family members *Mcl1* and *Bcl2l1* were also included because the effect of LIF and CHIR on ES cells could be in part attributed to the inhibition of apoptosis. Although I have already demonstrated that forced *Bcl2* expression does not support self-renewal (see chapter 4) its effect on apoptosis might still be relevant if it is regulated by the inhibitors.

When expression levels were examined in passage 3 cultures in LB, PS or 3i it was observed that expression levels of *Zfx* did not vary significantly but that *Tcl1* and *Tbx3* were significantly lower in PS (Fig5.11a). This is likely to reflect differentiation of the PS cultures as indicated by the down-regulation of pluripotency associated genes (Fig5.11a). Bcl-family members were not significantly altered with the exception of *Bcl2* which was expressed at higher levels in PS (Fig5.11a), the opposite of what might be expected given the increased apoptosis observed in PS cultures. The change may reflect increased *Bcl2* expression in differentiated ES cell derivatives. To assess the more immediate effects of CHIR cells were initially cultured in 3i before washing out the inhibitors and further culturing the cells in different conditions for 24 hours and analysing changes in gene expression. Down-regulation of the canonical Wnt target genes, *Axin2* and *Cdx1*, served as a control for the removal of CHIR (Fig5.11b, PS condition) while upregulation of the ERK target, *Egr1*, was as expected when PS was removed from the media (Fig5.11b, CHIR condition). Both *Tcl1* and *Tbx3* were down-regulated in PS as compared to 3i (Fig5.11b). However, when the cells were cultured in CHIR alone for the same 24 hour period *Tcl1* and *Tbx3* also showed down-regulation relative to 3i culture. It seems unlikely therefore that they are direct downstream targets of CHIR, rather, the change in their expression likely reflects early differentiation steps as evidenced by the upregulation of *Fgf5* (Fig5.11b). Similarly, *Zfx* levels were slightly lower in PS or CHIR than in 3i, perhaps indicating down-regulation upon initiation of

differentiation (Fig5.11b). Over the same time period there was little response of Bcl family members (Fig5.11b).

Finally, the response of the candidate genes to an 8 hour stimulation with CHIR was assessed. Known targets of canonical Wnt signalling, *Axin2*, *Cdx1* and *T*, were significantly upregulated (Fig5.11c) but none of the candidate genes showed any response over this time-frame with the exception of a small increase in *Tbx3* expression (Fig5.11c). I conclude from this analysis that while *Tcl1* and *Tbx3* are rapidly down-regulated upon the withdrawal of CHIR from 3i they do not show any direct response to CHIR and are unlikely to be significant downstream transcriptional targets in the promotion of self-renewal. No other changes were observed that correlate with the cellular phenotype.

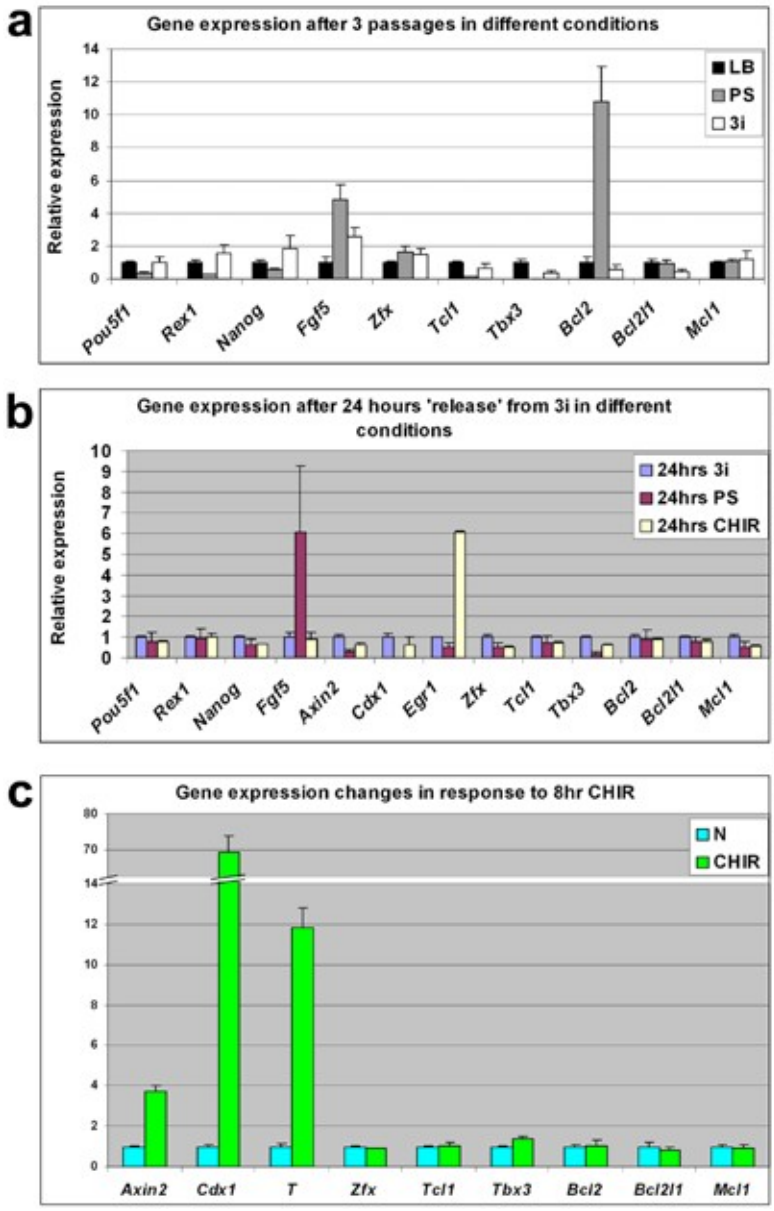


Fig5.11. Candidate responses to CHIR. a) Histogram showing relative gene expression in E14Tg2a ES cells after 3 passages in N2B27 plus LIF and BMP4 (LB); PS or 3i. b) Histogram showing relative gene expression in E14Tg2a ES cells. Cultures maintained in 3i had inhibitors washed out and were cultured for a further 24 hours in N2B27 plus 3i; PS; or CHIRON99021 (CHIR), 3μM before extracting RNA for analysis. c) Histogram showing relative gene expression in Oct4GiP ES cells after 12 hours' culture in N2B27 and a further 8 hours in N2B27 alone (N) or plus CHIR, 3μM. a,b,c) Gene expression was normalised to β-actin for each sample. Error bars represent standard deviation of the mean for 3 biological replicates.

5.3 Discussion

5.3.1 The effect of GSK3 inhibition on ES cells

I have provided evidence that inhibition of the FGF4-MEK-ERK pathway prevents differentiation of mouse ES cells (see chapter 4). This is in agreement with past observations including recent work showing that ES cells treated with pharmacological inhibitors of this pathway or genetically defective for components

of the pathway do not differentiate efficiently (Kunath et al., 2007; Stavridis et al., 2007). This was taken further by attempting long-term culture of ES cells in the absence of serum and growth factors with only PS. Our findings indicate that while this is possible at relatively high density, some differentiation is always evident and clonal propagation is not possible. The addition of CHIR to PS to create 3i fully rescues the properties of ES cell self-renewal considered essential for a growth condition to be described as 'sufficient' (see chapter 3). In this chapter I have attempted to identify the down-stream signalling pathways that mediate the effect of CHIR. As discussed in the introduction there are many known targets of GSK3 that affect many different processes. Pleiotropic effects of GSK3 inhibition were anticipated but it was hoped that a candidate approach might implicate or rule out a role for some down-stream targets in the regulation of self-renewal.

The phenotype of ES cells grown in serum-free conditions in the presence of CHIR together with other growth factors or inhibitors was examined. In CHIR alone self-renewing colonies persist for several passages, in marked contrast to N2B27 alone where rapid differentiation occurs and the majority of cells die upon passaging. It is also possible to establish ES colonies at clonal density in CHIR alone with high efficiency. These observations show that CHIR promotes short-term self-renewal but the accumulation of differentiated cells and deterioration of the culture with passaging clearly indicate that CHIR is not sufficient for long-term self-renewal. Differentiation is eliminated by the addition of LIF or of PS. I propose that, in the context of CHIR, LIF and PS function to prevent non-neural differentiation but leave in-tact the effects of CHIR that appear to promote the growth and survival of ES cells.

From previous work it is known that LIF can prevent the non-neural differentiation that occurs in serum or in the presence of BMP4 (Ying et al., 2003a) and it is likely that it functions in the same manner to prevent differentiation in the presence of CHIR. It follows that CHIR is fulfilling the role of BMP4 or serum by preventing the neural differentiation that normally occurs in LIF alone (Ying et al., 2003a). However, this is clearly not the only function of CHIR since ES cells grown in serum

or BMP4 in the absence of LIF undergo rapid differentiation and cannot be passaged, unlike the ES cells that persist in CHIR alone. Inhibition of the FGF4-MEK-ERK signalling cascade has been shown to prevent differentiation of ES cells along neural and non-neural lineages (Kunath et al., 2007). PS prevents differentiation from occurring by blocking this pathway (see chapter 4) so the differentiation that normally occurs in the presence of CHIR is prevented and efficient self-renewal proceeds. In summary, GSK3 inhibition has potent self-renewal effects if non-neural differentiation is otherwise blocked.

BMP4 was not able to prevent the spontaneous differentiation observed in CHIR. This was not surprising since the only clearly established role for BMP signalling in ES cell self-renewal is the inhibition of neural differentiation (Ying et al., 2003a). Notably, however, CHIR did prevent the rapid differentiation normally observed upon the addition of BMP4 to ES cell cultures. This indicates an effect of CHIR separate from inhibition of neural differentiation which is already effectively inhibited in the presence of BMP4. This phenomenon is analogous to the phenotype described for GSK3 double-knockout ES cells which exhibit resistance to multilineage differentiation (Doble et al., 2007) despite the high incidence of specific differentiation observed in standard culture conditions (see chapter 3).

5.3.2 Canonical Wnt signalling

The best studied effect of GSK3 inhibition is the activation of canonical Wnt signalling by stabilization of β-catenin. In fact, this effect is often considered to the exclusion of other GSK3 targets (Sato et al., 2004). I asked to what extent Wnt ligands could substitute for CHIR, reasoning that canonical Wnt signalling would be activated but other GSK3 targets would be unaffected. The addition of Wnt3a to PS enhances self-renewal but fails to recapitulate the phenotype of 3i. Notably, the neural differentiation observed in PS alone was not evident in the presence of Wnt3a. This is expected given the anti-neural effect of Wnt signalling on ES cell differentiation (Aubert et al., 2002; Haegele et al., 2003) and may in part explain the

promotion of self-renewal. However, a degree of non-neural differentiation is induced and proliferation is poor as compared to 3i. At this point we can speculate on how Wnt ligands might affect self-renewal and how their effect might differ from direct inhibition of GSK3. That a concentration of Wnt3a which elicited a similar TCF/LEF reporter response to 3μM CHIR was necessary to support self-renewal, and that activation of β-catenin seemingly recapitulates this effect strongly implicates canonical Wnt signalling downstream of Wnt3a. The activation of TCF/LEF target genes is a likely candidate for the downstream effect. In particular, Tcf3 is thought to act as a repressor of pluripotency regulators *Oct4*, *Sox2* and *Nanog* as well as many of their target genes, becoming a transcriptional activator upon activation of canonical Wnt signalling. However, work by Ying *et al* (Ying et al, in press) suggests that increased transcription of TCF/LEF target genes does not promote self-renewal in 3i. This implies TCF/LEF-independent effects of β-catenin which are much less well documented but are known to occur (reviewed in (Mulholland et al., 2005)).

5.3.3 Cell-cell adhesion

The morphology of cells grown in 3i, with tight-packed round colonies that frequently detach from gelatine-coated plates and grow as floating spheres, is distinct from cultures in serum plus LIF or LIF plus BMP4 which form flattened monolayers. This morphological change is dependent on CHIR (it is not observed in PS alone) and is recapitulated by the addition of Wnt3a or stable expression of β-catenin. *Ecad*-null ES cells grow as dispersed monolayers and do not form three-dimensional colonies. It seems likely that the morphological effect of CHIR is mediated through increased β-catenin incorporation into adherens complexes (Bienz, 2005) but the robust growth of *Ecad*-null cells clearly demonstrates that this is not essential for self-renewal in 3i. I have ruled out that 3i functions through adherens junctions but the lack of a response to Wnt3a in clonal assays suggests that Ecad may be required for the response to Wnt3a. If this is the case it suggests that increased cell-cell adhesion mediates the observed response of wild-type cells to Wnt3a.

Interestingly, *Ecad*-/- ES cells were more easily propagated in PD03 alone than wild-type cells (see chapter 4). The formation of colonies at clonal density was also more efficient. In the absence of a parental control line for the *Ecad*-/- ES cells this is difficult to interpret. It may simply be the case that the background of this cell line is more amenable to culture in the absence of LIF or CHIR. Alternatively, the absence of Ecad may alter the properties of the cells such that they become less dependent on cytokine stimulation and GSK3 inhibition. It is anticipated that in the absence of functional adherens junctions the amount of free β-catenin in the cytoplasm may increase (Bienz, 2005) and that this may result in higher basal levels of nuclear β-catenin and target gene activation. This could be tested by immunoblotting and TCF/LEF reporter assays and may prove informative in future attempts to define the role of canonical Wnt signalling in ES cell self-renewal.

5.3.4 Non-canonical Wnt signalling

Wnt ligands are known to activate β-catenin-independent signalling pathways (reviewed in (Kohn and Moon, 2005)), the best described of which is the planar cell polarity (PCP) pathway in *Drosophila*. A similar pathway is believed to operate in vertebrates resulting in activation of c-Jun N-terminal kinases (Jnk). This pathway may be activated by Wnt ligands but not CHIR. c-Jun is a component of the AP-1 transcriptional complex which activates IEGs and other targets downstream of MAPK signalling (reviewed in (Whitmarsh, 2007)). If its activation was in some way detrimental to self-renewal this could in part explain the relatively poor self-renewal promoted by Wnt3a as compared to CHIR. This is supported by the observation that Wnt3a but not CHIR induces expression of *Egr1*, *c-Fos* and *JunB* in a 1 hour acute response assay. The induction of these target genes is less pronounced than the response to LIF where they are likely responding to activation of ERK1/2 and it is unclear what the significance of this is likely to be for self-renewal. It would be informative to investigate the activation of β-catenin-independent pathways at the protein and transcriptional level in response to Wnt ligands and to assess their effect on self-renewal, perhaps by specifically blocking their activation. Alternative Wnt ligands may also elicit different responses if the balance in activation of canonical

Wnt signalling and PCP pathways is different. However, the fact that direct activation of 4OHT-inducible β-catenin does not recapitulate the phenotype observed with CHIR suggests that β-catenin activation is not sufficient and that negative effects of Wnt signalling are unlikely to be the sole cause of the differences in the effects of CHIR and Wnt3a.

5.3.5 Wnt-independent effects of GSK3 inhibition

Wnt ligands may activate signalling pathways not influenced by CHIR and the reverse is certainly true. Direct GSK3 inhibition leads to dephosphorylation (and usually activation) of many GSK3 targets (reviewed in (Doble and Woodgett, 2003)) as exemplified by NDRG1 (Murray et al., 2004). GSK3 negatively regulates translation by direct phosphorylation of eukaryotic translation initiation factor 2 (eIF2) (Welsh et al., 1998) whereas activated ERK promotes translation (Kleijn and Proud, 2000; Pyronnet, 2000). CHIR is anticipated to increase translation by derepression of eIF2 and this, together with other GSK3 targets, may increase the biosynthetic capacity of ES cells. This would explain the enhanced proliferation, viability and clonogenicity observed in the presence of CHIR. Furthermore, studies in yeast have suggested that biosynthetic capacity and 'pathway capacity', the efficiency with which signals are transmitted, are negatively correlated (Colman-Lerner et al., 2005). If this were true of ES cells cells with low biosynthetic capacity might be expected to have correspondingly high pathway capacity and to be more sensitive to signals that promote differentiation. In this hypothetical situation, ERK inhibition, as in PS, might, as well as inhibiting differentiation, have the paradoxical effect of lowering the threshold for differentiation by decreasing biosynthetic capacity. The addition of CHIR might therefore indirectly influence fate choice by raising biosynthetic capacity in the compromised context of PS. Other signalling pathways, including LIF-STAT3 and PI3K, might similarly influence biosynthetic capacity and in doing so promote self-renewal (see general discussion).

5.3.6 β-catenin as a filter of transcriptional noise

It has been suggested that Wnt-mediated stabilization of β-catenin can function as a 'filter' for transcriptional noise, functioning primarily to stabilize transcriptional programs rather than to activate transcription (Arias and Hayward, 2006). In the context of ES cell self-renewal β-catenin stabilization may act to reinforce the transcriptional program of ES cells. If this were done in a context where differentiation is largely prohibited, as in the presence of PS or of LIF, ES cells would be expected to be highly biased towards symmetrical self-renewal. β-catenin is proposed to mediate this function by recruiting chromatin modifiers to enhancer regions (Arias and Hayward, 2006) which presumably stabilize the activation status of the gene in question.

An emerging candidate that may promote or stabilize pluripotency is Tcf3. Tcf3 is the most abundantly expressed of the 4 TCF/LEF TFs in ES cells and its ablation leads to elevated Nanog levels and delayed differentiation (Pereira et al., 2006). A study showing that Tcf3 co-occupies many promoters with the pluripotency master regulators Oct4 and Nanog and that it acts to repress the transcription of *Oct4*, *Sox2* and *Nanog* as well as their target genes (Cole et al., 2008) provides a plausible mechanism for β-catenin's involvement in self-renewal. As Tcf3 acts as a transcriptional repressor in the absence of β-catenin and as canonical Wnt signalling is low in routine culture conditions (Ogawa et al., 2006) the effect of Tcf3 is to repress expression of pluripotency associated genes (Cole et al., 2008). Upon activation of β-catenin, however, Tcf3 becomes a transcriptional activator. Thus, canonical Wnt signalling may promote self-renewal by enhancing and/or stabilizing expression of the core pluripotency machinery. It would be interesting to investigate the effect of Wnt activation or Tcf3 knockdown on the homogeneity of Nanog expression. The mechanisms underlying the fluctuations in Nanog levels observed in self-renewing ES cells (Chambers et al., 2007) are not known but investigations into the effects of canonical Wnt signalling have thus far been restricted to population level analyses. The observation of increased Nanog expression observed in response to Tcf3 knockdown (Pereira et al., 2006) or Wnt activation (Cole et al., 2008) could

be explained by increased expression in individual cells or increased homogeneity of Nanog expression in the population.

It is clear then that many effects of GSK3 inhibition are anticipated and that they are expected to influence ES cells in different ways. I propose that it is a combination of effects that elicits the ultimate phenotype and that at least some aspects of the effect are independent of β-catenin. Activation of canonical Wnt targets that are associated with lineage commitment, such as *Brachyury* and *Cdx1*, may promote differentiation and may also be responsible for the observed inhibitory effect on neural differentiation. The striking effect on viability and proliferation makes it likely that there is an effect on biosynthetic capacity. Examining this will require further study beginning with an assessment of the activation status of GSK3 targets such as eIF2 which I propose to be involved. Assessing whether biosynthetic capacity influences fate choice is a rather more difficult task (see general discussion). Experiments addressing this problem in yeast required an elegant system of pathway responsive and non-responsive reporter genes that allowed variations in output in response to an input signal to be attributed to different sources, namely 'pathway capacity' and 'expression capacity' – the ability of cells to transcribe RNA and synthesise proteins (Colman-Lerner et al., 2005). It is conceivable that similar experiments could be performed in ES cells but it would be a large undertaking. Similarly, to attempt to assess the effect of β-catenin on transcriptional noise may require that reporter systems are devised that allow sensitive evaluation of multiple genes in single cells and variation in expression between individual cells (Arias and Hayward, 2006). While the obvious experiment is to undertake global transcriptional profiling of ES cells in 3i this is likely to be informative of the nature of their state but not of how that state is established or maintained.

5.3.7 cMyc

The manner in which cMyc expression is regulated and its proposed role in self-renewal (Cartwright et al., 2005b) made it an attractive candidate for our studies. It was found to be down-regulated in PS and it was suspected that this might in part

underlie the compromised viability and proliferation of these cells. However, the addition of CHIR or LIF, both of which promote dramatically enhanced cell growth, did not affect cMyc expression at the transcript or protein level. This is in marked contrast to published observations which claim that *cMyc* transcription is directly activated by STAT3 (Cartwright et al., 2005b). A critical omission in that study is that acute response of the *cMyc* gene was never tested. It is possible that activation of *cMyc* transcription by LIF occurs via activation of ERK signalling. This would be consistent with our observations of *cMyc* downregulation in PS. These results cast serious doubt on the importance of elevated cMyc in promoting self-renewal downstream of either LIF or GSK3 but to formally test this it would be necessary to culture ES cells lacking cMyc in 3i and, if possible, to obtain ES cells lacking both cMyc and NMyc to exclude redundancy.

5.3.8 Nanog

Nanog can be regulated indirectly by signals that promote self-renewal over differentiation because its expression correlates with the undifferentiated state of ES cells. It appears Nanog responds in this manner to GSK3 inhibition since CHIR can maintain ES cells short term and GSK3-DKO cells are resistant to differentiation (Doble et al., 2007) but no evidence was found for direct regulation of the transcript or the protein. Increased Nanog expression in 3i may result from decreased ERK activity (see chapter 4) and from the maintenance of a relatively pure population of ES cells**.** It has been proposed that β-catenin activation can increase Nanog transcription through an interaction with Oct4 (Takao et al., 2007). While this has not been investigated directly the lack of a change in Nanog levels after an 8 hour exposure to CHIR contradicts these findings. It will be interesting to investigate how CHIR affects the expression and activity of the core TFs, particularly in the context of β-catenin function as a filter for transcriptional noise (Arias and Hayward, 2006).

The phenotypes of Nanog-overexpressing and knockout ES cells clearly demonstrate that the effect of CHIR is not mediated through Nanog. Nanog-overexpressing cells retained a robust response to CHIR in the form of increased colony forming ability.

Interestingly, the majority of colonies formed from Nanog-overexpressing cells in CHIR alone had no overt differentiation, in contrast to wild-type cells. This is in agreement with our observations of ES cells in LIF plus CHIR or in 2i/3i (see section 5.3.1). Nanog overexpression presumably provides another means to inhibit the differentiation observed in CHIR alone resulting in robust self-renewal. *Nanog*-null ES cells also self-renew with increased efficiency in response to CHIR providing proof that this response is not Nanog-dependent. The fact that *Nanog*-null ES cells respond to CHIR similarly to wild-type cells is also informative. In both cases colonies of ES cells persist in cultures that are largely differentiated and ES cells can be expanded while cultures without CHIR become completely differentiated. If Nanog expression was required for the persistence of ES cells in CHIR alone it would be expected that *Nanog*-null ES cells would differentiate rapidly. However, this is not the case and it indicates that the pleiotropic response of ES cells to CHIR is not caused by heterogeneity of Nanog expression (Chambers et al., 2007) and a resulting difference in the response of subpopulations of cells within the culture.

5.3.9 Candidates

The view was taken that genes reported in the literature to have a role in promoting self-renewal might function downstream of CHIR to restore efficient growth and clonogenicity in the context of PS. At the very least these experiments allowed obvious candidates to be eliminated from further enquiries. While several of the candidates I chose to test were expressed at higher levels in 3i than in PS there was no evidence that they responded directly to CHIR with the exception of *Klf2*. The differences observed between PS and 3i likely reflect the down-regulation of the genes in differentiated cells present in PS cultures. Forced expression of *Klf4* could not recapitulate the effect of CHIR although it did appear to reduce differentiation. Similar results have been obtained for *Klf2* by colleagues (J. Hall, unpublished). As outlined in the introduction, CHIR was expected to have pleiotropic effects and as such it is not surprising that transcriptional targets that can recapitulate its effect have not been identified. However, this work is useful if only in the sense that it excludes the possibility that obvious candidates from the literature are responsible for the

phenotype observed. This is another situation where global transcriptional profiling might be informative, in identifying genes with significantly different levels of expression in ES cells cultured in 3i as compared to other conditions that support self-renewal. Caution must be exercised however because differences in the expression of many genes are anticipated that are incidental to the culture conditions and not in any way involved in promoting self-renewal. Furthermore, GSK3 is a kinase and its primary effects are at the level of protein function and stability. Inhibition of its activity, together with inhibition of ERK activity, is likely to have broad effects on the activity of proteins in ES cells with transcriptional changes secondary to protein regulation.

Chapter 6: General Discussion

6.1 Mechanism of ES cell self-renewal

In order to self-renew stem cells must undergo division without commitment to differentiate. ES cells cultured in permissive conditions will do this indefinitely, expanding rapidly in culture. Since they were first established the conditions for their growth have been progressively refined. Feeder cells can be replaced by LIF (Smith et al., 1988; Williams et al., 1988) and serum by defined media supplements and BMP4 (Ying et al., 2003a). Defined growth conditions make it possible to investigate the precise requirements for self-renewal and hence the molecular mechanisms governing self-renewal.

6.1.1 Regulation of self-renewal by intracellular signalling pathways

It is known that LIF acts through STAT3 to promote self-renewal (Niwa et al., 1998) and several downstream targets have been proposed to mediate this effect. *cMyc* (Cartwright et al., 2005b) and *Klf4* (Li et al., 2005) are both proposed to be direct transcriptional targets of STAT3. While overexpression experiments implicate both of them in the promotion of self-renewal the critical experiment would be to assess the response of ES cells lacking these genes to LIF. However, the situation is complicated by the effects of redundancy with both *cMyc* and *Klf4* being part of gene families, multiple members of which are expressed in ES cells. The work presented in chapter 5 casts doubt on whether *cMyc* is indeed a target of STAT3 and shows that ES cells retain a robust response to LIF without upregulating cMyc. Forced Klf4 expression appeared to promote self-renewal but efficient self-renewal continued to require the addition of LIF or CHIR demonstrating that upregulation of Klf4 is not the only important effect of LIF. In fact, the only gene described to date capable of supporting robust cytokine-independent self-renewal is Nanog (Chambers et al., 2003) and there is no evidence that Nanog is directly regulated by the growth factors

considered essential for self-renewal. On the contrary, ES cells overexpressing Nanog retain a response to LIF (Chambers et al., 2003) and *Nanog*-null cells can be derived and maintained in standard growth conditions, reliant on the provision of LIF (Chambers et al., 2007). Only in the case of BMP signalling has it been shown that a single transcriptional target, *Id* genes, can substitute for the growth factor (Ying et al., 2003a). In short, although LIF-STAT3 signalling is accepted to be a critical pathway for ES cell self-renewal no single target has been identified that can substitute for its function.

The work described in chapter three shows that this 'critical' pathway is in fact dispensable for ES cell self-renewal. In 3i culture conditions STAT3 is inactive and ES cells have been derived in 3i that are genetically deficient for *Stat3*. It has previously been suggested that ES cells can self-renew independently of LIF signalling (Dani et al., 1998) but never before has independence from STAT3 been formally demonstrated. It is possible that the loss of STAT3 is compensated for by other members of the STAT family but the observation that *Stat3*-/- ES cells differentiate in LIF-dependent growth conditions indicates that STAT3 is critical for self-renewal. Clearly, it is possible to achieve inhibition of differentiation, with proliferation, through STAT3-independent means. It is possible that LIF/STAT3- and CHIR-activated signalling converge on a common target(s) that mediates their effects and drives self-renewal. An alternative explanation is that propagation of ES cells can be achieved through different signalling pathway manipulations with no core set of signalling targets absolutely required for self-renewal.

Conceptually, the self-renewal of ES cells can be broken down into two parts. Firstly, cell fate decisions. At each division a cell can "choose" to self-renew or to differentiate. The activation status of signalling pathways will influence this choice. Secondly, cell survival and proliferation. The composition of the media and again the status of signalling pathways will influence viability and growth rate. Under hypothetical, optimized culture conditions no differentiation would occur and the cells would proliferate rapidly with no cell death. In reality ES cell cultures always contain a proportion of differentiated cells and often exhibit a degree of cell death.

Considering the phenotype of ES cells in 2i/3i culture and their behaviour in PS or PD03 and in CHIR alone in the context of this framework I propose that ERK inhibition biases cell fate choices in favour of self-renewal (Burdon et al., 1999b; Kunath et al., 2007) while LIF or CHIR serve to activate pathways that increase ES cell survival and proliferation. This fits reasonably well with our observations: when ERK activity is reduced differentiation is suppressed but cell proliferation and viability are poor until LIF or CHIR is added when viability and proliferation are restored. However, several observations suggest that this is an over-simplification.

In LIF alone ES cells undergo neural differentiation (Ying et al., 2003a) but differentiation is decreased as compared to cells cultured without LIF (see chapter 4). If LIF had no effect on fate choice it would be expected that neural differentiation would proceed at a similar frequency. Furthermore, as shown in chapter 4 ES cells maintained in either LIF or PS alone undergo neural differentiation. This indicates that neither condition is capable of imposing a total block on neural differentiation. However, when combined LIF and PS support robust self-renewal including efficient colony formation from single cells. Therefore, two culture conditions, independently insufficient to suppress neural commitment completely, prevent neural differentiation when combined.

In CHIR alone, non-neural differentiation proceeds but again ES cells persist through multiple passages and colonies are even observed at clonal density. GSK3 inhibition diverts ES cells from neural differentiation so it is clearly capable of influencing fate choice but why is the differentiation incomplete, why do a proportion of cells continue to self-renew? Similarly to LIF, combining CHIR with PS or PD03 almost totally eliminates differentiation despite the fact that neither component separately is capable of preventing differentiation. In an analogous manner to LIF and BMP4, ERK inhibition could prevent non-neural differentiation (Chan et al., 2003) while CHIR prevents neural differentiation (Aubert et al., 2002; Haegele et al., 2003), the two combining to limit the options for differentiation available to ES cells. However, several observations suggest that this is not the complete explanation. Firstly, ERK inhibition does not exclusively prevent non-neural differentiation, as reported

(Kunath et al., 2007; Stavridis et al., 2007), and as observed when BMP4 was combined with PS (see chapter 4). Secondly, BMP4 cannot replace CHIR in this context. When combined with PS non-neural differentiation becomes evident in place of neural differentiation. Thus, elimination of residual neural differentiation is not sufficient to turn PS or PD03 into efficient self-renewal conditions. Thirdly, in chapter 5 activation of canonical Wnt signalling by addition of Wnt3a was observed to prevent neural differentiation but did not support robust self-renewal when combined with PS or PD03. Finally, CHIR has the dual effect of promoting expansion of ES cells while causing non-neural differentiation. This is inconsistent with the idea that GSK3 inhibition might slot exclusively into either an "inhibition of differentiation" or "enhancement of viability and proliferation" role.

So how can these observations be reconciled? It seems that the separation of fate choice and cell proliferation/viability is an inadequate model for ES cell behaviour. The phenotypes observed could be explained if LIF and CHIR raised the threshold for signal activation required to induce ES cells to commit to differentiate (Silva and Smith, 2008). In such a model ERK inhibition might become sufficient to prevent differentiation along any lineage when the threshold for differentiation is raised by the addition of LIF or CHIR. At present this is speculative given the lack of experimental data to address such a hypothesis. However, it has previously been shown that the metabolic status of a cell and its response to environmental stimuli are correlated.

An experimental system in yeast was developed (Colman-Lerner et al., 2005). Fluorescent reporter genes were placed under the control of either a promoter that responded to activation of the mating-type response pathway upon exposure to α-factor or constitutively expressed, α-factor-independent promoters. Input to this model system was α-factor and output expression of the α-factor-dependent reporter. Differences in the level of reporter expression between individual cells were modelled as arising from differences in two 'subsystems'. The pathway subsystem comprised everything required to transmit the pheromone signal to activation of the TF Ste12. The expression subsystem comprised everything required for expression of

the reporter in response to activation of the TF ie. transcription and translation. The capacity of these two subsystems was seen as dependent on the number, localization and activity of proteins that transmit the signal (pathway capacity) or express genes into proteins (expression capacity). Cell-to-cell variation in expression capacity could be measured from differences in expression from α-factor-independent promoters and this could be correlated with variation in the expression from α-factor-responsive promoters. At lower α-factor concentrations the variation due to pathway capacity was found to increase but the total variation in system output did not increase. This implies that expression capacity and pathway capacity are negatively correlated. If similar mechanisms are employed in ES cells "expression capacity" might be increased in the presence of LIF or CHIR leading to a reduction in "pathway capacity" and a decreased sensitivity to signals that promote differentiation (Silva and Smith, 2008). The expression capacity or biosynthetic capacity of ES cells is expected to be lower in the absence of LIF or CHIR and may be lowered further still by inhibition of ERK signalling. This may explain why, despite a block of differentiation imposed by PS or PD03, ES cells cultured under these conditions become heterogeneous. Note that the data presented in chapter 4 demonstrate that inhibition of apoptosis by forced expression of *Bcl2* is not sufficient to convert ERK inhibition into a robust condition for self-renewal. This demonstrates that simply increasing viability cannot reproduce the effect of LIF or CHIR. Rather, I propose that regulatory input is required from these molecules to increase biosynthesis (Fig6.1). If ES cell self-renewal was regulated by global mechanisms interlinking metabolic and growth status with sensitivity to differentiation-promoting stimuli it would not be surprising that attempts to identify targets of LIF-STAT3 that promote self-renewal have not yielded a target that fully recapitulates the effect of LIF. Similar difficulties are expected to affect the search for the mediators of the effect of GSK3 inhibition (see chapter 5).

The phenotype of ES cells cultured in the absence of insulin may also be directly relevant to this hypothesis. In the absence of insulin proliferation is extremely slow and viability is decreased. In this condition the expression capacity of ES cells might be decreased and they might therefore be more sensitive to differentiation cues. A

testable hypothesis is that removing insulin from the media prior to induction of differentiation might lead to more rapid and/or homogeneous differentiation.

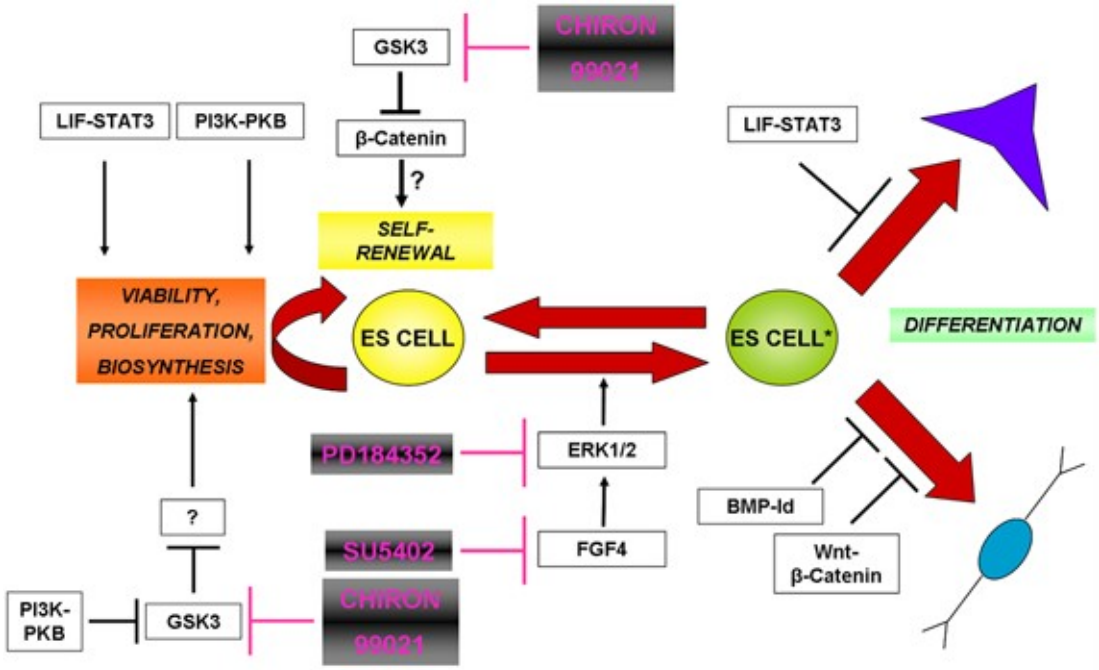


Fig6.1. A model for ES cell self-renewal. Self-renewal results from a combination of inhibition of differentiation and promotion of autonomous replication by increasing biosynthesis. Differentiation can be inhibited by imposing lineage-specific blocks on differentiation through activation of LIF-STAT3, BMP-Id and Wnt-β-catenin signalling pathways. Differentiation can also be inhibited by shielding ES cells from the effects of ERK activity which promotes responsiveness of ES cells to differentiation cues. This can be achieved with a combination of FGFR and MEK inhibitors. Self-renewal is promoted by signals enhancing cell survival, proliferation and biosynthesis. PI3K-PKB signalling promotes proliferation and inhibits apoptosis. LIF-STAT3 signalling and inhibition of GSK3 may affect prolferation and viability directly but also increase biosynthesis which increases the threshold for differentiation. GSK3 inhibition may also affect self-renewal through stabilisation of β-catenin.

6.1.2 Signalling pathways and the core transcription factors

How does signalling relate to regulation of the core TFs Oct4, Sox2 and Nanog? Genetic knockout experiments have demonstrated that the expression of these three TFs is essential for establishment and maintenance of pluripotency in ES cells and in the embryo (Nichols et al., 1998; Niwa et al., 2000; Chambers et al., 2003; Mitsui et al., 2003; Masui et al., 2007), with the exception of the recent report that Nanog is dispensable for the maintenance of established ES cells although levels of Nanog expression profoundly influence the efficiency of self-renewal (Chambers et al.,

2007). It seems reasonable to suppose that these critical TFs will be regulated at some level by the signals that are known to promote self-renewal. However, there is scant evidence for direct regulation of the core TFs downstream of extracellular signalling molecules. A notable exception is canonical Wnt signalling. Emerging evidence that Tcf3, a putative effector of Wnt signalling in ES cells, regulates transcription of *Oct4*, *Sox2* and *Nanog* (Pereira et al., 2006; Cole et al., 2008) implies direct regulation of pluripotency by an exogenous signal. However, it is significant that activation of canonical Wnt signalling is not sufficient to maintain pluripotency, which requires simultaneous activation of LIF-STAT3 (see chapter 5) (Ogawa et al., 2006). In this thesis I elaborate on previous work implicating blockade of, as opposed to activation of, a signalling pathway in promoting self-renewal. Preventing activation of ERK1/2 inhibits differentiation of ES cells (Burdon et al., 1999b; Kunath et al., 2007), allowing self-renewal to proceed. Thus, the emerging picture is that deflection of differentiation-promoting signals, rather than instructive regulation of pluripotency, is the key effect of manipulation of extracellular signals.

If positive signalling input is not required to promote expression of the core pluripotency TFs they must be autonomously expressed while the cells remain undifferentiated. This situation is thought to be stabilized by the ability of the TFs to activate their own and one another's transcription (Tomioka et al., 2002; Boyer et al., 2005; Kuroda et al., 2005; Okumura-Nakanishi et al., 2005; Rodda et al., 2005). How the core transcriptional machinery maintains pluripotency is not precisely known but the development of techniques permitting global analysis of transcription, promoter occupancy and protein interactions has produced a wealth of data relating to this subject. Chromatin immunoprecipitation analyses revealed that Oct4, Sox2 and Nanog co-occupy a large number of promoters (Boyer et al., 2005; Loh et al., 2006). Microarray transcriptional analyses revealed that the genes whose promoters are occupied by these TFs can be grouped into actively transcribed and inactive genes (Boyer et al., 2005). Thus, the core transcription factors, presumably in combination with other transcriptional regulators, appear to employ both positive and negative gene regulation in order to maintain the pluripotent transcriptome. A recent investigation of the promoter occupancy of 9 TFs associated with pluripotency

suggests that promoters bound by a single TF are inactive while those occupied by multiple members of the pluripotency machinery are actively transcribed (Kim et al., 2008). This implies coordinate regulation of gene expression by the key TFs. Actively transcribed genes were found to be enriched for pluripotency-associated genes including *Oct4*, *Sox2* and *Nanog* themselves, genes associated with proliferation, components of signal transduction machinery, and chromatin modifiers and are presumed to promote self-renewal. Silent genes are enriched for TFs associated with lineage commitment and it is thought that the recruitment of polycomb repressive complexes (PRCs) to these genes is involved in their silencing (Boyer et al., 2006). The idea that the core TFs may regulate gene expression through chromatin modifiers was supported by the finding that the proteins may interact with complexes including NuRD and SWI/SNF (Wang et al., 2006). It appears that the pluripotency-associated TFs regulate both the expression and the activity of machinery capable of influencing the transcriptional profile of ES cells. However, ES cells are remarkably tolerant of loss of function mutations affecting the epigenetic machinery (Montgomery et al., 2005; Schoeftner et al., 2006; Pasini et al., 2007) indicating that it is direct transcriptional control by the core TFs that is essential for maintenance of pluripotency (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003; Masui et al., 2007).

It has been proposed that if ES cells are shielded from differentiation cues the pluripotency network is self-sustaining and ES cells undergo a programme of autonomous self-replication (Ying et al, in press). If this is the case then differentiation would be promoted by destabilization of this network. The finding that levels of Nanog expression fluctuate in the ES cell population and that low levels are associated with an increased propensity to differentiate (Chambers et al., 2007) suggests that Nanog is the obvious candidate for regulation by differentiation-promoting signals. Indeed, it has been reported that ERK signalling negatively regulates Nanog expression (Hamazaki et al., 2006) and the data presented in chapter 4 corroborates this although whether regulation is direct remains to be seen. However, loss of Nanog does not lead to inevitable differentiation (Chambers et al., 2007) and does not therefore reflect commitment to differentiate in wild-type cells.

Furthermore, *Nanog*-null cells remained sensitive to the self-renewal promoting effects of MEK inhibition (see chapter 4) demonstrating that the promotion of differentiation by ERK signalling is not mediated through negative regulation of Nanog (at least not exclusively).

6.1.3 ERK Signalling and commitment to differentiate

ERK activation promotes responsiveness to differentiation cues and I model the activation of ERK1/2 by FGF4 as a reversible step in the initiation of lineage commitment (Fig6.1). The step is clearly reversible as ES cells cultured in routine conditions (serum plus LIF or LIF plus BMP4) have constitutively active ERK but remain responsive to MEK inhibition when culture conditions are altered. It will be informative to investigate how ERK activity impinges on the components of the pluripotency network to promote differentiation. It has been suggested that lineage-associated transcription fluctuates in ES cells at levels below the threshold that would initiate differentiation. In self-renewing ES cells the transcriptional program directed by Oct4, Sox2 and Nanog remains dominant. ERK signalling may increase the global expression of lineage-associated genes or it may act directly on targets that increase the sensitivity of ES cells to signals promoting differentiation (Silva and Smith, 2008). In chapter 4 I discussed the possibility that the function of ERK activity is to promote open chromatin conformation, perhaps through the downstream kinases MSK1/2. This provides a plausible mechanism for increased expression of lineage-associated genes but there is no evidence that the transcriptionally permissive chromatin of ES cells is dependent on ERK and, as discussed above, ES cells are tolerant of loss of epigenetic silencers.

If the effect of ERK signalling is global it may be difficult to identify the critical targets but predictions can be made about the effects of decreased ERK activity. The level of transcription of lineage-associated genes may be reduced and this could be tested by transcriptional profiling. The degree of variation between individual cells, in terms of genes expressed, may also be reduced when ERK activity is lower. Preliminary results suggest that Nanog is expressed more homogeneously in ES cells

cultured in 2i/3i conditions than in LIF plus BMP4 or serum plus LIF (T. Kalkan, unpublished).Tthe same could be true of other markers expressed in ES cells including Rex1 which is known to be expressed at different levels in individual ES cells (Toyooka et al., 2008). Furthermore, transcriptional profiling of isolated single cells (Kurimoto et al., 2006) grown in different culture conditions might reveal differences in the degree of cell to cell variation. Such experiments are technically demanding and a large number of individual cells would have to be profiled before variation could be examined statistically. However, the status of individual ES cells and the degree of variation across the population has significant implications for controlling differentiation. If, as suggested by the work presented here and by others (Kunath et al., 2007), ES cells proceed through an ERK high stage to differentiate then ES cells cultured in standard culture conditions where ERK is constitutively active (see chapter 3) are closer to the point of commitment than ES cells in culture conditions where ERK activity is low such as 3i. EpiSCs are reliant on provision of FGF for self-renewal and therefore have constitutively active MAPK signalling. ES cells cultured in standard conditions may contain EpiSC-like intermediate cells, perhaps increasing the heterogeneity of the population. The response of ES cells to differentiation cues is likely to depend on the status of the cells, in terms of signalling pathway activation and gene expression. The homogeneity of differentiation may be increased by increasing the homogeneity of the starting ES cell population.

6.1.4 Pluripotency and the cell cycle

Pluripotent cells have a unique cell cycle (Savatier et al., 1994). Constitutively active Cdk2/CyclinE and corresponding low pRb activity is thought to underlie the short G1 and rapid entry of ES cells to S-phase (Stead et al., 2002). Upon differentiation the G1 phase becomes longer and cell cycle-dependent control of CyclinE expression and Cdk2 activity is established (White et al., 2005). This coincides with the observation of cell-cycle dependent activity of pRb family members and regulation of E2F target genes. The simplest interpretation of these observations is that elaboration of cell cycle-dependent Cdk2 activity and G1/S control follows

differentiation of pluripotent cell into somatic cell types. However, the detailed dynamics of the change in cell cycle regulation have not yet been elucidated and it remains to be clearly shown that loss of pluripotency precedes these cell cycle changes. It remains a formal possibility that the development of G1/S control actually precedes differentiation. If it was necessary to develop G1/S regulation before differentiation could initiate then signals promoting progression through the cell cycle might prevent differentiation simply by preventing this from occurring. In this regard, it would be interesting to investigate how, if at all, the cell cycle of EpiSCs differs to that of ES cells. EpiSCs are undifferentiated but correspond to a later developmental stage than ES cells. If they have a functional G1/S checkpoint it would suggest that elaboration of G1/S control precedes differentiation of ES cells.

Several lines of evidence indirectly suggest that signals promoting passage through the cell cycle may promote self-renewal (Niwa, 2007b). cMyc was reported to be a target of LIF-STAT3 signalling and its forced expression was apparently sufficient to sustain LIF-independent self-renewal (Cartwright et al., 2005b). cMyc is known to promote progression through the cell cycle through transcriptional activation of genes involved in cell cycle regulation (reviewed in (Hooker and Hurlin, 2006)). The PI3K pathway is also known to promote cell proliferation. Mutation of *Pten*, which negatively regulates PI3K, increases the proliferation rate of ES cells (Sun et al., 1999) while loss of *Eras*, which positively regulates PI3K, results in decreased proliferation (Takahashi et al., 2003). Several reports have implicated activation of this pathway in the promotion of self-renewal (Paling et al., 2004; Storm et al., 2007) with one report going so far as to claim that activated Akt was sufficient for self-renewal of both mouse and human ES cells (Watanabe et al., 2006). RNAi-knockdown of *Tcl1*, a PKB co-factor, also promoted differentiation but its forced expression was not enough to prevent differentiation (Ivanova et al., 2006). While none of these studies attributed the observed effects to regulation of the cell cycle it is possible that the phenotypes observed are at least partly attributable to changes in cell cycle regulation. Evidence for the effect of direct perturbation of the cell cycle on self-renewal is scant. Inhibition of Cdk2 activity (Stead et al., 2002) or of E2F-activated transcription (White et al., 2005) has been reported but no details were

given as to the effect on self-renewal. This suggests that no dramatic effect was observed but fails to address the question of whether imposition of cyclin periodicity promotes differentiation. Indeed, these experiments may be difficult given the apparent resistance of ES cells to attempts to down-regulate Cdk activity (Stead et al., 2002; White et al., 2005).

To examine the possibility that differentiation requires prior establishment of G1/S control would be difficult. It would require close monitoring of cell cycle dynamics during differentiation with respect to a precisely defined point of commitment beyond which the ES cell state is irreversibly lost. This is particularly difficult because differentiation does not occur synchronously in all cells within a population. It would be interesting to see if differentiation became more homogeneous and synchronous when ES cells were synchronized with regard to the cell cycle. This would imply that cell cycle dynamics influenced differentiation although it would not directly address the relationship between cell cycle control and differentiation.

6.2 An in vivo role for FGF-ERK signalling

The work described in this thesis hinges on the inhibition of the FGF-MAPK signalling pathway, in contrast to the activation of this pathway employed by addition of FGF in EpiSC culture (Brons et al., 2007; Tesar et al., 2007). As expected, EpiSCs cannot be maintained in 2i/3i. The relevance of the FGF signalling pathway for pre-implantation development is becoming increasingly clear and provides a clear rationale for the effectiveness of FGFR- and MEK-inhibitors in enhancing ES cell self-renewal. FGF4 is expressed in the early embryo (Rappolee et al., 1994) together with FGFR2 (Arman et al., 1998). The most informative phenotype described to date is the failure of *Grb2*-/- embryos to form the Gata6-positive primitive endoderm (Chazaud et al., 2006). Primitive endoderm precursors are distinguished from epiblast precursors at E3.5 by reciprocal expression of Gata6 and Nanog. Lineage tracing experiments suggest that cells at this stage are committed to one lineage or the other. *Grb2*-/- embryos homogeneously express Nanog in all cells of the ICM at this stage indicating a failure to specify the primitive

endoderm. In wild-type embryos by E4.5 a morphologically distinct primitive endoderm is observed on the surface of the ICM and the primitive endoderm and epiblast can be distinguished by reciprocal expression of Gata4 and Nanog. Incubation of 8 cell embryos to the blastocyst stage in 3i/2i prevents formation of the primitive endoderm (J. Silva and J. Nichols, unpublished). The phenotype is recapitulated by PS or PD03 alone with the addition of CHIR seemingly supporting formation of a larger ICM. Culturing embryos which have already formed the primitive endoderm in 3i does not eliminate the primitive endoderm indicating that these cells are not killed by the inhibitors. It seems therefore that pharmacological blockade of ERK activation also prevents primitive endoderm specification. The requirement for this signalling pathway in primitive endoderm specification is mimicked in ES cells. ES cells lacking *Fgf4* (Wilder et al., 1997), *Shp2* (Qu and Feng, 1998) and *Grb2* (Cheng et al., 1998) have all been described and reported to have defects in differentiation. The work described in this thesis elaborates on this work and demonstrates that ERK inhibition is critical to preservation of the pluripotent state in the absence of LIF and BMP4. *In vivo* phenotypes described for *Fgf4*- (Feldman et al., 1995) and *Fgfr2*- (Arman et al., 1998) knockouts are also consistent with the successful formation of a pluripotent epiblast but subsequent compromises to development resulting from a failure to form extraembryonic tissues and/or a requirement for ERK signalling in tissues of the embryo proper. Similarly, embryos lacking ERK2 died at the early post-implantation stage with defects in trophoblast development but formed normal ICM outgrowths, indicating successful establishment of the epiblast (Saba-El-Leil et al., 2003). It would be interesting to re-examine the phenotypes of these knockout embryos in light of the recent descriptions of preimplantation phenotypes. If FGF4 is the key signalling molecule involved in primitive endoderm specification I predict that the FGFR-inhibitors SU and PD17, used alone, will produce the same phenotype as PS or PD03, prevention of primitive endoderm specification. The combination of studies suggested above should give a clear idea of the role of FGF4, produced by cells of the ICM, in driving differentiation of the pluripotent epiblast. It appears that following specification of primitive endoderm cells in the E3.5 embryo they are sorted into a morphologically distinct layer, probably as a result of differential expression of cell adhesion

molecules (Chazaud et al., 2006). Understanding why some cells but not others become specified will be an interesting avenue for further investigation. The responsiveness of ES cells and ICM cells to differentiation cues is likely to be conserved and understanding one is likely to shed light on the other.

6.2.1 "Capturing Pluripotency"

Expression of *Fgf4* is under the control of Oct4 and Sox2 (Yuan et al., 1995). Thus, critical TFs in the regulation of pluripotency promote the expression of a growth factor that drives differentiation. Work presented here and by Kunath *et al* (Kunath et al., 2007) shows that this signal promotes responsiveness to differentiation cues. Critically, FGF4 does not promote lineage-specific differentiation. This implies that *in vivo* pluripotent epiblast cells may be destabilized by ERK activity downstream of FGF4 before becoming further specified by signals within the environment of the embryo. This effect is predicted to be mediated during a narrow developmental window corresponding to the embryonic stages from which ES cells can be derived. Beyond this the pluripotent epiblast becomes dependent on FGF signalling and is no longer responsive to ES cell growth conditions instead becoming responsive to FGF and activin (Brons et al., 2007; Tesar et al., 2007). It is not yet clear if intermediate stages exist or what their properties are. Deriving ES cells requires that epiblast cells are prevented from progressing along their normal developmental pathways. Our experiments on ES cells, and the responsiveness of the embryo to 3i/2i culture conditions (Ying et al, in press), show that inhibition of ERK activation is an effective way of achieving this (Fig6.1). If early developmental pathways are conserved across mammals it should be possible to 'capture' cells at a corresponding pluripotent stage to that represented by mES cells (Silva and Smith, 2008).

ES cells are distinct from somatic cells in their independence from ERK signalling and the lack of G1/S cell cycle control (Stead et al., 2002). The cells of the early epiblast appear to share these properties and it has been suggested that it is the autonomous self-replication of ES cells and their *in vivo* counterparts that underlies their inherent tumorigenicity (Silva and Smith, 2008). Both ES cells and epiblast

cells can give rise to teratocarcinomas when placed in a permissive site in immuno-deficient mice (reviewed in (Chambers and Smith, 2004)). These common properties seem to suggest that ES cells are not an *in vitro* phenomenon but an *in vivo* state captured by permanently removing them from the inductive environment of the developing embryo.

6.3 Implications of 3i

6.3.1 Alternative culture conditions highlight new possibilities

Chapter three, together with recent work (Ying et al, in press), describes a novel culture condition for the propagation of ES cells *in vitro* – 3i. The media was first developed by examining the effects of pharmacological inhibitors alone and in combination on the growth of established ES cell lines, as judged by morphology and growth rate. Optimisation by trial and error led to the development of a media that passed the "tests" normally applied to growth conditions to establish that they are in fact sufficient to maintain ES cells. Clonal propagation informed us that cell-cell interactions were not required. *De novo* derivation proved that prior adaptation to *in vitro* culture was not necessary to allow ES cells to respond to this media. Germ line transmission of ES cells derived and propagated in 3i showed that the cells remained truly pluripotent and karyotypically normal. These findings bring 3i in line with all other established growth conditions for ES cell culture and go a good deal further than reports that claim to have established "sufficient" or "defined" conditions. This in itself is interesting but it soon became apparent that these conditions were quite different in their properties from those previously described. Firstly, it was possible to establish germ-line competent ES cells from CBA strain mice (Ying et al, in press), the first time this has been reported without the use of feeders and serum. Previous success in the derivation of ES cells from CBA mice was achieved by microdissection (Brook and Gardner, 1997) or by using a MEK inhibitor (PD98059) (Buehr and Smith, 2003) together with conventional derivation protocols. The success of microdissection may be the result of completely isolating the epiblast

from the other tissues of the blastocyst which may be a source of differentiation-promoting signals that standard culture conditions are not able to overcome. MEK inhibition presumably functions to prevent the epiblast cells from initiating differentiation in response to those same signals. 3i similarly employs a block of ERK signalling. Secondly, it is clear that LIF and BMP signalling are not required when ES cells are cultured in 3i. These differences suggested that there are multiple ways to derive and maintain ES cells. More importantly, it offers fresh impetus to attempt the derivation of ES cells from other species where the conditions established for mouse have so far failed.

As discussed above, there is good evidence that preimplantation embryos respond in a similar manner to ES cells to 3i/2i. The most exciting implication of this is its possible application to the derivation of 'true' human ES cells as opposed to the EpiSC-like lines already in existence. In fact, our group has already established rat ES cells in 3i conditions that contribute to chimeras when reintroduced to the developing embryo (Buehr et al, submitted). This property is critical in distinguishing the cells from EpiSCs which do not contribute to chimeras (Brons et al., 2007).

6.3.2 Differentiation

The attraction of ES cells lies in their ability to provide an *in vitro* model for early development and in their potential clinical applications as an unlimited source of cells for research and cell replacement therapies (Smith, 2001). Realising their potential requires that we understand their biology and develop protocols for their differentiation into specialized cell types (Murry and Keller, 2008). Currently, differentiation protocols yield heterogeneous populations of cells, with only fraction of cells developing into the desired cell type. It is not clear why an apparently homogeneous population of genetically identical cells should give rise to heterogeneous populations when induced to differentiate. Aside from the existence of a fraction of committed or differentiated cells within ES cell cultures, it is becoming apparent that ES cells are heterogeneous. A striking example of this is the

fluctuations in levels of Nanog (Chambers et al., 2007). It is reasonable to suppose that in the presence of such variation the population will initiate differentiation asynchronously and may enter distinct programs of differentiation. It may therefore be necessary to devise strategies to increase the homogeneity of the starting population and to assess the effects of ES cell culture conditions on subsequent differentiation. In this context 3i provides an alternative to established conditions as a starting point for differentiation. Furthermore, our observations suggest that the population in 3i is more homogeneous with regard to the low level of background differentiation and with regard to Nanog expression (T. Kalkan, unpublished). This may provide a better platform for directed differentiation although this has yet to be assessed. ES cells divide rapidly and asynchronously in culture and position in the cell cycle may also affect initiation of differentiation. Strategies designed to synchronize the population before beginning differentiation protocols might also help to direct homogeneous differentiation.

6.3.3 3i and Reprogramming

The recent demonstration that terminally differentiated cells can be induced to acquire pluripotency by the introduction of 4 TFs (Takahashi and Yamanaka, 2006) has taken stem cell research one step closer to the goal of patient-specific cell therapies. However, several major problems must be overcome before iPS cells can be used in treatments for humans.

In most cases reported for the generation of mouse iPS cells a selection strategy was used based on the expression of antibiotic resistance genes from pluripotency-associated promoters (Takahashi and Yamanaka, 2006; Okita et al., 2007; Wernig et al., 2007). This can only be done using cells that have previously been genetically modified and is not therefore applicable to human cells. Ideally iPS cells would be generated without the need for selection strategies based on genetic modifications. Morphological changes can be used as an indicator of the acquisition of pluripotency (Meissner et al., 2007) but cells with ES-like properties have been observed that do not contribute to chimaeras (Takahashi and Yamanaka, 2006) and are not therefore

truly pluripotent. Furthermore, it is as yet unclear what effect, if any, the activation of intracellular signalling pathways has on the generation of iPS cells. The culture conditions in which cells are grown prior to or after introduction of the TFs may influence the efficiency of generation of iPS cells and, more importantly, the status of the iPS cells generated.

So, how does 3i relate to these problems of iPS cell generation? 3i could provide a method for selection of iPS cells from a heterogeneous population. Somatic cells and EpiSCs are normally dependent on active ERK signalling (Brons et al., 2007; Tesar et al., 2007) so the inhibition of this pathway is likely to lead to cessation of proliferation and/or cell death in these populations, permitting expansion only of cells which have acquired an ES cell phenotype. This would remove the need for selection strategies based on genetically modified starting populations and would provide a more stringent selection than morphology alone. While morphological criteria will not exclude EpiSC-like populations and cells that have undergone incomplete conversion such as those generated by *Fbx15* selection (Takahashi and Yamanaka, 2006), the ability to grow in 3i (as opposed to serum and LIF) is anticipated to exclude these populations (Silva and Smith, 2008).

An exciting possibility is that different ES cell culture conditions might actively influence the conversion to pluripotency. As yet, it is not known how each of the 4 TFs acts to convert the somatic cell genome to pluripotency. Culture conditions may influence the activity of signalling pathways that have similar effects to the activity of the TFs themselves. Certain signals may influence the activity of epigenetic modifiers or enhance the proliferation of cells undergoing conversion. A potential effect of 3i/2i in this regard is an enhancement of the expression of Nanog which appears to be negatively regulated by ERK signalling (see chapter 4). This would be anticipated to enhance the conversion of pre-pluripotent ES cells, represented by those ES-like cells which express only low levels of Nanog and Rex1 and do not contribute to chimaeras (Takahashi and Yamanaka, 2006), to fully pluripotent ES cells. It has been suggested that activation of the endogenous Nanog gene is a critical step in the conversion to truly pluripotent iPS cells (Silva and Smith, 2008). Direct,

side-by-side comparisons of different ES cell medias for the derivation of iPS cells and subsequent examination of the properties of the cells generated will reveal if the culture conditions do in fact influence the process of conversion to pluripotency.

6.4 Concluding Remarks

Since they were first derived in 1981 ES cells have been employed in the study of early developmental decisions and for the generation of transgenic mice. The significance of this was recognised with the award of the Nobel Prize for Physiology or Medicine to Martin Evans, together with Mario Capecchi and Oliver Smithies, for developing the methods that would allow genetic modifications to be introduced to mice. The field of stem cell research continues to grow, primarily because of the belief that stem cells will one day provide a limitless source of cells for cell replacement therapy. This goal may be some way off but stem cells may also be used to generate clinically relevant cells for *in vitro* drug screening.

ES cells are capable of generating any cell type of the adult organism. It is theoretically possible therefore to generate any cell type *in vitro*. This makes ES cells a particularly powerful tool. However, there are still significant limitations to our understanding of ES cell biology and to our ability to control their differentiation. A major concern is that attempts to establish ES cells from other species which have a phenotype corresponding to mouse ES cells have so far failed. The human ES cells currently in existence are more similar to mouse EpiScs than they are to mouse ES cells. The derivation of 'true' human ES cells would facilitate the translation of research from the mouse model and provide another pluripotent cell type with distinct properties that may prove useful in future attempts to utilize these cells in studies of development, in drug screening or in cell replacement therapy.

The development of 3i ES cell culture conditions and the corresponding effects on the behaviour of the cells of the inner cell mass provide a rationale for the existence of ES cells and may provide a means to 'capture' pluripotency in other species. Furthermore, an increased understanding of the mechanisms controlling ES cell self-

renewal should facilitate their controlled differentiation and therefore their biomedical application.

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